PURIFICATION AND cDNA CLONING OF HUMAN PLACENTAL 11ß-HYDROXYSTEROID DEHYDROGENASE.

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DECLARATION.

I declare that this thesis and the work presented here are entirely the result of my own independent investigation. Where I received assistance this is acknowledged in the text and on the following page.

This work has not been and is not concurrently submitted for any other degree.

I received assistance with the following aspects of the work presented in this thesis:-

1.11ß-HSD2 amino acid sequence was obtained on an amino acid sequencer maintained and run by Douglas Lamont and based at the WELMET facility, Department of Biochemistry, University of Edinburgh.
2.The synthetic 11ß-HSD2 peptide was synthesised by Dr Bala Ramesh, (Department of Protein and Molecular Biology, Royal Free School of Medicine, Rowland Hill Street, London.) and was coupled to carrier by Dr Brian McGinn, Thistle Peptide Services, Department of Biochemistry, Glasgow University.

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Abstract

11ß-hydroxysteroid dehydrogenase efficiently inactivates potent glucocorticoid hormones (cortisol and corticosterone), leaving aldosterone unmetabolised. Abundant 11ß-HSD activity in human placenta plays a central role in controlling fetal glucocorticoid exposure, which if excessive is harmful and may predispose to low birth weight and hypertension in adulthood. Similar 11ß-HSD activity in the distal nephron protects mineralocorticoid receptors from glucocorticoids and appears important in normal blood pressure control Loss of this protection results in hypertension : the syndrome of apparent mineralocorticoid excess (SAME). In previous work an NADP-dependent isoform of 11ß-HSD has been purified from rat liver, a cDNA isolated and the human homologue cloned. For sometime the gene encoding this isoform (11ß-HSD1) was believed to give rise to the 11ß-HSD activity responsible for the crucial 'protective' 11ß-HSD activities in placenta and kidney. However, some evidence suggested there may be tissue-specific 11ß-HSD activities that could not be explained by the 'liver-type' isoform. In particular, 'liver-type' 11ß-HSD antigen appears absent from renal DCT and the hepatic enzyme's relatively low affinity for cortisol and corticosterone suggest a limited effectiveness in clearing these glucocorticoids.

The work in this thesis began by partially purifying the 11ß-HSD activity from human placenta and comparing it to that from rat liver. The activities differed substantially in all parameters measured and did not merely reflect species differences as rat placental 11ß-HSD was similar to the human placental isoform. This clearly showed, for the first time that human tissues contained an 11ß-HSD isoform (which we designated 11ß-HSD2) distinct from that encoded by the previously known 'liver-type' isoform (11ß-HSD1). Moreover this 11ß-HSD2 isoform was an exclusive dehydrogenase and kinetic measurements indicated it had over one hundred fold higher affinity for glucocorticoids and thus was much better suited to the tissue-specific eradication of glucocorticoids attributed to the crucial 'protective' 11ß-HSD activities in placenta and kidney.

Purification of human placental 11ß-HSD2 16000-fold, to homogeneity, was achieved by subcellular fractionation, detergent solubilisation, AMP-affinity chromatography and 2-D electrophoresis. Placental 11ß-HSD2 is ≈40 kDa protein, with a basic pI and is N-terminally blocked. Over 100 residues of internal amino acid sequence was obtained by digestion of the homogeneous protein and sequencing of several of the resulting tryptic peptides. Purification was assisted by a novel technique allowing highly specific (single spot on 2-dimensional

electrophoresis) photoaffinity labelling of active 11ß-HSD2 in crude tissue extracts by its glucocorticoid substrates. This work reveals 11ß-HSD2 is a novel member of the short chain alcohol dehydrogenase superfamily (apparent monomer M_r 40000). It is a very basic (apparent pI = 9.1) intrinsic membrane protein requiring, as yet undefined, membrane constituents for full stability. Affinity chromatography and affinity labelling studies suggest 11ß-HSD2 has a compulsory ordered mechanism, with NAD binding first, followed by conformational change allowing glucocorticoid binding with high affinity.

Amino acid sequence from homogeneous human placental 11ß-HSD2 was used to isolate an 1897bp cDNA encoding this enzyme (predicted M_r 44126, predicted pI 9.9). Transfection into mammalian (CHO) cells produces 11ß-HSD2 activity which is NAD-dependent, without reductase activity, avidly metabolises glucocorticoids (K_ms for corticosterone, cortisol and dexamethasone of 12.4 1.5, 43.9 8.5 and 119 15nM respectively) and is inhibited by glycyrrhetinic acid and carbenoxolone (IC_{50} 10-20nM). Rabbit antiserum recognising 11ß-HSD2 has been raised to an [11ß-HSD2 residues 370-383]-carrier conjugate. Recombinant 11ß-HSD2, like native human placental 11ß-HSD2, is detectable with affinity labelling and 11ß-HSD2 antiserum, and appears to require little post-translational processing for activity. 11ß-HSD2 mRNA (1.9kb transcript) is expressed in placenta, aldosterone target tissues (kidney, parotid, colon and skin) and pancreas. *In situ* hybridisation and immunohistochemistry localise abundant 11ß-HSD2 expression to distal nephron in human adult kidney and to trophoblast in placenta. 11ß-HSD2 transcripts are expressed in fetal kidney (but not lung, liver or brain at 21-26weeks), suggesting an 11ß-HSD2 distribution resembling that in adult is established by this stage in human development.

Thus the work presented in this thesis describes the identification of a new 11ß-HSD isoform (11ß-HSD2), methods for its highly specific photoaffinity labelling and purification to homogeneity followed by the isolation and sequencing of tryptic peptides and a full-length cDNA and raising of a specific antibody. Tissue localisation of the 11ß-HSD2 activity, enzyme protein and mRNA all confirm it to be the isoform responsible for the crucial protective 11ß-HSD activities in placenta and kidney. Moreover several researchers have now shown that mutations in 11ß-HSD2 are indeed the cause of hypertension in SAME and now 11ß-HSD2 is regarded by many as a candidate gene contributing to the causation of essential hypertension.

List of Figures and Tables.

Commonly Used Abbreviations.

11ß-HSD	11ß-hydroxysteroid dehydrogenase
2-D[electrophoresis]	2 dimensional electrophoresis
(c)AMP	(cyclic) adenosine monophosphate
ANOVA	analysis of variance
bp	base-pair
CAPS	3[cyclohexylamino]-1-propanesulphonic acid
CBG	cortisol binding globulin
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1- propanesulphonate
CHO[cells]	Chinese hamster ovary cells
CMC	critical micelle concentration
DAB	3,3'-diamino benzidine tetrahydrochloride
DEAE	diethylaminoethyl
DHEA(S)	dehydroepiandrosterone (sulphate)
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethelenediaminetetraacetic acid
GR	glucocorticoid receptor
HPLC	high performance liquid chromatography
IEF	isoelectric focusing
IUGR	intrauterine growth retardation
kb	kilobases
K_m	Michaelis constant
K_d	dissociation constant

MR	mineralocorticoid receptor
mRNA	messenger RNA
NAD(H)	nicotinamide adenine dinucleotide (reduced form)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced form)
NEPHGE	non-equilibrium pH gradient gel electrophoresis
PBS	phosphate-buffered saline
PVDF	polyvinylidene difluoride
PVP-40	polyvinylpyrrolidone (average mol. wt. 40000).
RNA	ribonucleic acid
SAME	syndrome of apparent mineralocorticoid excess
SCAD	short chain alcohol dehydrogenase
(SDS)-PAGE	(sodium dodecyl sulphate) polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
TEMED	N,N,N',N'-tetramethylethyldiamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
UV	ultraviolet

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Chapter 1 : Introduction

1.1 Glucocorticoids, mineralocorticoids and their receptors

Glucocorticoids and mineralocorticoids are synthesised in the adrenal cortex and are collectively known as corticosteroids. Glucocorticoids are very potent (normal, unstressed, human plasma cortisol ranging 50-600nM) and have a very large number of diverse effects throughout the body, being of particular importance at times of stress and in modulating the body's fuel metabolism and immune and inflammatory responses[149]. Aldosterone is the principal mineralocorticoid in most species, including humans and rodents. Aldosterone is also a very potent hormone, circulating levels being in the high picomolar range (100-1000 fold lower than levels of total glucocorticoid), but its actions are largely restricted to homeostasis of fluid and electrolyte balance and to the control of blood pressure, and are exerted through its actions on a limited number of 'target tissues' (notably the distal nephron of the kidney).

In the late 1960s and early 1970s the receptors for corticosteroids were identified by binding studies[21,140,142]. The type II corticosteroid receptor bound glucocorticoids with high affinity, was a poorer binder of aldosterone[23] and was almost ubiquitously distributed amongst tissues. This fitted very well with the actions of glucocorticoids and the receptor became known as the glucocorticoid receptor or GR. In contrast, the type I corticosteroid receptor bound aldosterone with very high affinity and so could be expected to be responsive to the relatively low levels at which this hormone circulates[21,150]. Moreover, the type I receptor had a much more restricted tissue distribution, which included the target tissues of aldosterone[20,150]. These findings fitted very well with the actions of aldosterone and the type I corticosteroid receptor also became known as the mineralocorticoid receptor or MR. A type III corticosteroid receptor was also described originally and seemed to bind corticosterone with highest affinity (corticosterone > cortisol > progesterone > aldosterone > dexamethasone) [22] and was particularly abundant in the kidney. The exact nature of this 'type III binder' has not been completely resolved, but the evidence points to it not being a receptor through which the actions of corticosteroid hormones are mediated[98], unlike GR and MR which are widely accepted as the mediators of the vast majority of the genomic actions of corticosteroid hormones[149].

Although, it had been known that glucocorticoids could bind MR and that aldosterone had some binding to GR[21,23], the overall picture that mineralocorticoids acted through MR and glucocorticoids through GR was sufficiently compelling that relatively little attention was paid to this incongruous binding. In 1983 however, a careful study of binding came to the conclusion that MR bound aldosterone and cortisol with approximately the same affinity[8]. This paradox came into sharp focus in 1987 when the cDNA for MR was cloned and the recombinantly expressed receptor protein did indeed bind cortisol and aldosterone with equal affinity and moreover bound the glucocorticoid corticosterone with the highest affinity of all[18]. Another study showed that *in vivo*, despite circulating at much lower levels, aldosterone was nonetheless gaining access to MR in preference to glucocorticoids in aldosterone 'target tissues' (e.g. distal nephron), though not in other MR containing tissues such as the hippocampus[34]. *Thus, there seemed to be an unexpected tissue-specific regulation of access to MR.* Initially, this was proposed to be due to a form of intracellular glucocorticoid binding protein[29]. However, the real mechanism turned out to be due to local inactivation of glucocorticoids by an enzyme 11ß-hydroxysteroid dehydrogenase (11ß-HSD), which is the subject of this thesis.

1.2 11ß-Hydroxysteroid dehydrogenase.

11ß-HSD catalyses the conversion of physiological glucocorticoids (cortisol and corticosterone) into their inactive 11-dehydro products (cortisone and 11-dehydrocorticosterone, respectively). 11ß-HSD enzyme activity had been known about since the 1950s [151,152] and has been reported present in many organs in the adult, placenta and fetus in humans and in a large range of animal tissues investigated [52]. Its distribution included kidney, but was certainly not restricted to aldosterone target tissues. It was also evident that the reverse, 11ß-reductase, reaction was present in some tissues, notably liver, but the tissue distribution of this activity was less clear and 11ß-reductase was known to be a relatively labile enzyme activity [31]. It was unclear how this 11ß-reductase related to the 11ß-HSD dehydrogenase activity.

Although the existence of 11ß-HSD activity was well documented, its physiological relevance was obscure. It had been reported to be deficient in association with a rare disorder known as the syndrome of apparent mineralocorticoid excess (SAME) in which there is severe

hypertension, sodium retention and hypokalaemia [75]. In 1985 the investigation of a unique adult patient showed that the disorder was due to novel renal mineralocorticoid actions of cortisol [99]. Thus, the apparent renal mineralocorticoid excess and hypertension could be reversed by suppressing endogenous cortisol with dexamethasone, and recreated by concurrent infusion of physiological doses of cortisol. This work and imaginative studies with liquorice [33], which was shown to inhibit 11ß-HSD, led to the suggestion that normally 11ß-HSD in the distal nephron inactivated cortisol and that if this enzyme was deficient (either congenitally as in SAME or acquired as in liquorice ingestion) then cortisol gained access to MR in the distal nephron and caused mineralocorticoid hypertension. Thus, it was proposed that 11ß-HSD was the tissue-specific regulator of access to MR [16,17].

In 1988, 11ß-HSD was purified from rat liver [35] and specific antisera were raised [35,38], an encoding cDNA cloned [36] and the human homologue isolated [41]. Over expression of the 11ß-HSD cDNA in mammalian cells finally proved this one enzyme was capable of NADPH-dependent 11ß-reductase activity as well as NADP-dependent 11ß-dehydrogenase activity [40]. As 11ß-HSD was proposed to have such an important role in the distal nephron, efforts were made to localise the 11ß-HSD protein and mRNA in kidney. Immunohistochemistry showed 11ß-HSD in kidney, but in proximal not distal nephron [14,17]. Activity staining of rat kidney appeared to reveal 11ß-HSD activity in the distal nephron utilising the cofactor NAD rather than NADP, but only with an unusual substrate (11-hydroxyandrostenedione), rather than the expected substrates corticosterone or cortisol [50].

Thus by 1991, the time when the work in this thesis commenced, there was good evidence to suggest 11ß-HSD activity was important in determining MR specificity although it was not definitively proven. It was however, very unclear whether the 11ß-HSD isolated from liver was responsible and this lead to much speculation about other 11ß-HSD variants or even a second NAD-dependent isoform [47,50,52]. 11ß-HSD activity had been documented in many other tissues not regarded as aldosterone 'target tissues', including placenta where the enzyme was clearly able to affect the glucocorticoid exposure of the developing fetus. Interest in placental 11ß-HSD began to grow rapidly following the rapidly accumulating evidence for a link between the in utero environment, reduced birthweight and adult disease especially hypertension.

1.3 Placental 11ß-HSD and the link between the in utero environment, birth weight and adult disease especially hypertension.

11ß-HSD activity was first reported to be present in placenta in 1960[153] and placental 11ß-HSD has since been described in several species[19,27,51,52,138,144,153]. The majority of reports are clear that the net activity in placenta is 11ß-dehydrogenase i.e. inactivating glucocorticoids. Although some *in vivo* work in baboons suggests that the predominant activity at mid-gestation is 11ß-reductase, by term it is clearly 11ß-dehydrogenase and all work in humans supports placental 11ß-HSD being predominantly 11ß-dehydrogenase, not just at term but throughout gestation. Thus work, based on:- *in vitro* enzyme assays, *ex vivo* perfusion studies and results of both of the *in vivo* radioactive tracer studies carried out during human pregnancy[24,139], all support the net activity in human placenta being 11ß-dehydrogenase. A very high level of 11ß-HSD activity has been reported in placenta from several species and in human placenta in particular. It has been calculated that the glucocorticoid-inactivating activity of human placental 11ß-HSD is sufficient to account for the much lower glucocorticoid levels in the fetal circulation[32]. Although other mechanisms may assist in the partitioning of active glucocorticoid at much lower concentrations in the fetal compartment (e.g. maternal-fetal gradients of glucocorticoid binding proteins, CBG[25] and albumin[143]) the evidence for these is conflicting and it is widely accepted that placental 11ß-HSD activity seems the major factor in human placenta close to term.

In human and in many animal species studied, fetal glucocorticoid levels are much lower (3-10 fold lower in human pregnancy) than maternal levels. Thus there seems a need, conserved in evolution, to limit fetal glucocorticoid exposure during at least part of gestation. This led to the idea that glucocorticoid exposure of fetal tissues is of importance in normal development and that placental 11ß-HSD plays a key role in regulating these levels and thus in promoting normal development. Certainly it is clear that excess fetal glucocorticoid exposure is harmful. Many studies have shown high dose glucocorticoids during pregnancy can be teratogenic in animals[4,5,7,124-126,128,131]. In humans this seems less certain, but with high dose maternal glucocorticoid treatment there are reports of teratogenesis[1]. In human pregnancy a clearer risk of glucocorticoid treatment is fetal intra-uterine growth retardation (IUGR) resulting in reduced birth weight for gestation[3]. Glucocorticoids are involved in many

processes during development. For example, glucocorticoids are essential for the normal structural development of organs such as the adrenal medulla and lung[6]. Glucocorticoid levels have a powerful influence on the maturation of many important fetal and neonatal homeostatic systems such as:- the handling of carbohydrate fuels by the liver[120-123,127,130]; the level of erythropoetin in the kidney[134]; the feedback responsiveness of the hypothalamic-pituitary-adrenal axis[135,136,141]; many aspects of the development of the sympathetic nervous system from early migration of neural crest and determination of neural crest cell fate[6,129,132,137] to transmitter phenotype[154,155] and tissue responsiveness at the receptor[79] and post-receptor levels[78,118,119]. Many of these glucocorticoid influences on fetal tissue maturation seem likely to give rise to permanent consequences if deranged. It is well established in work on rats that disordered steroid hormone action during development can lead to life-long consequences. For example, brief neonatal exposure to sex steroids lead to permanent neuroanatomical, neurochemical and behavioural changes which persist throughout adult life[156]. Similarly, a single neonatal testosterone injection has been shown to irreversibly programme the level of several enzymes (especially 5α and 5ß-reductases) [157]. Some effects of in utero steroid action may programme aspects of physiology in a fashion which gives the offspring an increased vulnerability to certain diseases much later in life. An example in humans being diethylstilboestrol exposure of the developing female fetus and greatly increased risk of vaginal cancer many years later in these daughters[158]. Thus there is good evidence that the level of fetal glucocorticoid exposure affects not only the rate of fetal growth and birth weight but also the structural development of organs and the maturation of homeostatic systems. Such developmental effects of steroid hormones are likely to have permanent consequences on the offspring's physiology (fetal programming) and may affect their lifetime vulnerability to certain diseases. There was thus considerable interest in the work of Professor David Barker, who in 1990[159] reported an association from human epidemiology in the UK (since verified by many studies[165]), showing a strong link between low birth weight and the risk of hypertension in adulthood.

The first clues of a link between early life events and major common 'Western diseases' (such as ischaemic heart disease, stroke and chronic bronchitis) came from studies showing an association between the maternal, perinatal and infant mortality rates in a region and the incidence of such common 'Western diseases' in adults originally from that region, whether or

not they migrated away from their birthplace[160,161]. These clues led to Barker's remarkable retrospective cohort follow-up studies on populations born in Preston and Hereford[81,165]. In these centres, excellent records of obstetric and perinatal morphological measurements were found and over 15000 people who were referred to in such birth records were traced, then all being in middle age or older. Correlations were then possible between characteristics at birth and subsequent risk of common adult diseases. Many striking findings emerged from these studies and it was clear that birth weight and body shape at birth were strongly linked to, and so predictive of, the individual's risk of hypertension[165]. The smaller the birth weight: the higher the risk of adult hypertension[159]. This strong relationship persisted when common confounding factors such as prematurity and social class had been controlled for. Moreover, the relationship held not just for extremes of birthweight but was continuous throughout the normal ranges of birth weight and blood pressure. Two types of low birth weight baby seemed predisposed to hypertension[163]. Firstly, a long thin baby, with a reduced head circumference and low ponderal index (weight/[length]^3); such babies tended to have small placentas and were predisposed to hypertension in association with syndromeX (dyslipidaemia, insulin resistance and non-insulin dependent diabetes mellitus) [163,164]. Secondly, babies that were disproportionately short for their head size; such babies tended to have large placentas and were predisposed to hypertension in association with elevated plasma fibrinogen[162,163,165]. These striking findings strongly implicated in utero events resulting in low birth weight for gestation in the causation of human hypertension. Two studies which began around the time of commencement of this thesis clearly implicate glucocorticoid action in utero in this link. Firstly, Benediktsson *et. al.*. showed placental 11ß-HSD in rats correlated with birth weight[59,133]. Two other studies, in humans, have reached the same conclusion.[71,166] Secondly, Lindsay *et. al.*. showed that administration of dexamethasone to pregnant rats throughout pregnancy resulted in offspring with significantly reduced birthweight (using a dose of dexamethasone causing no significant change in maternal weight gain during pregnancy compared to untreated normal controls) [80,166]. Moreover, the offspring then developed hypertension in adulthood. These studies support the hypothesis [58] that fetal glucocorticoid exposure in utero programmes fetal physiology in a manner that reduces birthweight and predisposes to adult hypertension.

Since placental 11ß-HSD is a major determinant of fetal glucocorticoid exposure this enzyme was clearly important and merited much closer study. Moreover, almost immediately on beginning the work of this thesis it became apparent that placenta contained 11ß-HSD activity with many differences from the NADP-dependent 11ß-HSD isoform cloned from liver. Early findings(see Chapter 4) suggested the placental 11ß-HSD activity was due to a second isoform with properties consistent with it being similar to the activity expected in the distal nephron of the kidney; in other words the sought after tissue-specific protector of MR thought deficient in the human hypertensive condition SAME [17]. This added a second very compelling reason for close study of the 11ß-HSD enzyme activity in human placenta in the expectation it would help in understanding the pathophysiology of human hypertension.

1.4 Aims.

The work in this thesis had the following aims in studying human placental 11ß-HSD:-

1) To characterise and isolate the major 11ß-HSD activity from human placenta.

2) To purify human placental 11ß-HSD to homogeneity and obtain amino acid sequence.

3) To clone a cDNA encoding the enzyme responsible for the 11ß-HSD activity in human placenta.

4) To raise a specific antibody against human placental 11ß-HSD.

Chapter 2 : Materials and Methods.

2.1 Materials

[1,2,6,7 ^{3}H] corticosterone, cortisol, aldosterone and [1,2,4,6,7 ^{3}H] dexamethasone (specific activity 78, 73, 80, and 84 Ci/mmol respectively), ^{35}S-methionine (specific activity >1000 Ci/mmol), ^{35}S- and ^{32}P-labelled nucleotides, Hybond N and Hybond ECL membranes, Hyperfilm $B_{max.}$, Sequenase v2 and the ECL Western blotting systems were purchased from Amersham International (Little Chalfont, U.K.). Autodigestion resistant modified trypsin and S.Aureus V8 protease were obtained from Promega (Southampton, UK) and hydrogenated Triton X100 (RTX-100, protein grade) from Calbiochem (Nottingham, UK). Glycerol and protein standards for SDS-PAGE (#44264L), electrophoresis grade SDS and urea were purchased from BDH Laboratory Supplies (Poole, Dorset UK). Coomassie blue dye concentrate, standardised bovine serum albumin and 2-D electrophoresis protein standards (#1610320) were purchased from Bio-Rad (Hemel Hempstead, UK). HPLC grade methanol and water were purchased from Rathburn Chemicals (Walkerburn, UK) and Quickszint 302 HPLC scintillant from Zinsser Analytic (Maidenhead, UK). Entensify fluoroautoradiography solutions were obtained from Du Pont/NEN (Stevenage, UK). PVDF (Problott) membranes were obtained from Applied Biosystems (Warrington, UK). AscI was obtained from New England Biolabs (Hitchin, U.K.). PGEM-11zf( ) plasmids, micrococcal nuclease-treated canine pancreatic microsomes (#Y4041), and other restriction enzymes were obtained from Promega (Southampton, U.K.). Synthetic oligonucleotides were synthesised by Oswel DNA Service (Edinburgh, U.K.). 1kb ladder DNA size markers, Lipofectin, media and reagents for tissue culture were purchased from Gibco/BRL (Paisley, U.K.). Vectastain® *Elite* ABC and DAB reagent system for immunohistochemistry was purchased from Vector Laboratories (Peterborough U.K.). Affinity chromatography matrices, Pharmalytes, detergents and other chemicals (both reagent and molecular biology grade) were obtained from Sigma Chemical Co. (Poole, Dorset, UK).

2.2 Buffers.

Tissue processing and chromatography buffer systems used were as follows:- Buffer A; 20% glycerol, 5mM potassium phosphate pH7.0, 1mM EDTA, 1mM dithiothreitol. Buffer B; 10%

glycerol, 300mM sodium chloride, 4mM CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1- propanesulphonate), 1mM EDTA, 0.02mM Tris-HCl pH 7.7 (buffer B2; sodium chloride omitted). Buffer C; 10% glycerol, 300mM sodium chloride, 1 mM EDTA, 0.02 mM Tris-HCl pH 7.7. Where pH was varied buffers included 10% glycerol, 1mM EDTA, 300mM sodium chloride and 0.1M buffer:- pH 5-6, potassium acetate; pH 6.5-7.5, potassium phosphate; pH 7.5-9, Tris-HCl; pH 9.5-10, potassium carbonate and pH 10.5-11, glycine-NaOH. Buffers were pH adjusted at the temperature of use. Where the buffer varies from that above, the variation is appended in [brackets].

2.3 Tissues and tissue processing.

Human term placentae (400-600g, normal vaginal delivery), were rapidly placed on ice and tissue processed within 2h. Adherent membranes, umbilical cord and large vessels were removed. Placental tissues were rapidly minced with scissors, washed in ice cold 0.9% saline, blotted dry, suspended in approximately 3 times their weight of buffer A and homogenised with a commercial blender. Homogenate was filtered through 2 layers of muslin and the filtrate fractionated rapidly by differential centrifugation:- (i) 10 min x 750g, (ii) 40 min x 25 000g and (iii) 60 min x 110 000g. The supernatant from each centrifugation was subjected to the next centrifugation finally leaving cytosolic supernatant. Rat (fasted Wistar adult animals killed by decapitation) and other human tissues were handled and fractionated similarly though because the amount of tissue was usually much smaller than for human placenta homogenisation was with Dounce or Ystral homogenisers (Scientific Instrument Centre, UK) and to aid fractionation, the homogenate was then diluted twofold (with buffer A), rather than being filtered so as to minimise losses from the small tissue quantities. Fractions resulting from tissue processing were frozen at -80^{o}C, or used immediately. When 11ß-HSD activity was labile (e.g. rat placenta) 11ß-HSD assays were always performed immediately.

2.4 Assays of 11ß-HSD activity.

C-11 dehydrogenase activity was determined by measuring the rate of conversion of 1.12×10^{-8}M [^{3}H]-steroid substrate (corticosterone or cortisol) to product (11-dehydrocorticosterone or cortisone respectively) in the presence of 400µM NAD or NADP (unless otherwise stated),

and calculating the percentage conversion during the assay. The 250µl assay consisted of:- 10µl containing tritiated steroid, 50µl containing cofactor and 190µl enzyme in 0.1M potassium phosphate pH 7.5, 300mM sodium chloride buffer (buffer only in blank controls). Reactions were incubated at 37°C for 10 min and terminated by adding 2ml ice-cold ethyl acetate and mixing. The organic layer was separated, evaporated and the steroid resuspended in 0.6ml 50% methanol:50% water. 0.2ml of this was injected into a Berthold HPLC system fitted with a Waters Associates µBondpack C18 column, which was eluted with 50% methanol:50% water v/v (at 1.8 ml/min for corticosterone substrate reactions). Eluted steroids were monitored by a UV absorbence detector and a Berthold LP506 C1 scintillation counter to detect tritiated steroids. The latter was flushed with Quickszint 302 scintillant (at twice the column elution rate) and output calibrated against known authentic steroid standards. The percentage conversion of steroid substrate to product was calculated as an index of enzyme activity. Cofactor concentrations and buffer pH were varied in experiments specifically examining these effects. The assay pH was verified by measuring the pH of a parallel mixture containing unlabelled steroid.

Protein was estimated by the method of Bradford [82] using Bio-Rad protein dye and calibration against standards of bovine serum albumin. Preliminary work identified optimum assay conditions for each tissue so that the amount of protein added (i) was in the linear region of the curve of protein concentration versus percentage substrate converted with 400µM of the more active cofactor (NAD or NADP) and (ii) resulted in a percentage conversion of 10-40% in 10 min. All experiments had blank (no protein) assays run in parallel to establish the background level of steroid product. 11ß-reductase activity was measured using the same reaction conditions except that 400µM NADH, or NADPH, and [^{3}H]-11-dehydrocorticosterone were used as substrates. The latter was prepared from [^{3}H]-corticosterone by a variation of the method of Lakshmi and Monder[35] using human placental enzyme with NAD at pH 7.5. Kinetic parameters were calculated from the initial velocity determinations in assays giving less than 30% conversion.

2.5 Detergent solubilisation of 11ß-HSD activity.

All solubilisation was carried out at 0-2°C. Tissue fractions, resuspended in buffer C to 6mg protein/ml, were mixed with an equal volume of solubilisation buffer (in buffer C with

detergent at twice final concentration). After 30 min, the mixture was centrifuged at 110 000g for 1 h. Supernatant containing soluble enzyme was carefully removed.

A screen was performed using 13 detergents of diverse classes[90], at concentrations of twice and half their critical micelle concentrations (CMC), see Chapter3 for details. Following this screen, conditions were refined for solubilising human placental 11ß-HSD in the most promising detergent (CHAPS). When comparison was made with rat liver 11ß-HSD, 0.06% Triton X100 (final detergent/protein ratio of 0.2) was used as this was found optimal for solubilising microsomal rat liver 11ßHSD (in agreement with Lakshmi *et. al.*.[30]).

2.6 Affinity Chromatography.

Affinity chromatography matrices were hydrated, loaded into simple columns and equilibrated in buffer B. Affinity matricies were screened for useful purification of NAD dependent 11ß-HSD using 1 ml bed-volume columns. The CHAPS-solubilised tissue fraction containing 11ß-HSD activity was loaded onto the column which was then washed with buffer B. 11ß-HSD was eluted with nucleotide cofactor in buffer B as indicated in the text. 1.5ml fractions from the column were collected, placed on ice and rapidly assayed for 11ß-HSD enzyme activity. In some experiments larger columns (5ml affinity matrix) were used and a 0-3000µM elution gradient of NAD, AMP or NADP in buffer B2 was applied across a total of 10 column volumes (50ml) After completion of the gradient the column was washed with buffer B and any remaining 11ß-HSD eluted with buffer B[3mM NAD]. This protocol was designed to determine thresholds of elution with different cofactors allowing their affinity for the active site of the enzyme to be compared [84,178]. Having identified the most useful affinity matrix preparative and analytical protocols were developed which maximised yield and purity respectively.

For the preparative 5'AMP-agarose chromatography, six 5ml columns were run in parallel, having a common outflow and collecting 5ml fractions. CHAPS-solubilised placental 25000g pellet fraction (180ml) was loaded (2.4ml/min) onto the columns, then washed with 205ml buffer B and 45ml buffer B[0.25M NaCl], and 11ß-HSD eluted with 170ml buffer B[0.2M NaCl + 1mM NAD], all at 3.5 ml/min. Fractions were placed on ice and rapidly assayed for 11ß-HSD enzyme activity. Fractions with abundant 11ß-HSD2 were pooled and concentrated (Amicon stirred cells, Centricon 10 concentrators, acetone precipitation).

Analytical work used 1ml columns run manually (collecting 1-1.5 ml fractions). For highest purity of eluting 11ß-HSD an N6-5'AMP-agarose column was washed with Buffer B (15ml), Buffer B[0.02M NaCl + 0.4mM NADH](10ml), Buffer B (15ml) then eluted with Buffer B[0.125M NaCl + 1mM NAD]. The flow rate was 420µl/min, reducing to 200-300µl/min on elution. The increased purity was at the cost of lower yield and reproducibility than the preparative protocol (above).

2.7 Removal of NAD from affinity purified 11ß-HSD.

NAD was removed from partially purified 11ß-HSD enzyme (eluted as above) by 3 successive filtration/dilution cycles in a Centricon-10 concentrator (Amicon). Following filtration by centrifugation at 4600g for 90 min, samples were diluted in buffer B and the cycle repeated. Removal of NAD was verified by measuring A_{280}. During this process samples were assayed for 11ß-HSD activity to determine dependence on added cofactor.

2.8 Statistical Analysis of Enzyme Activity Results.

Results of enzyme activity are shown as mean ± standard error. Series of paired samples were compared by one-way analysis of variance (ANOVA) of their difference. Kinetic parameters were estimated by Lineweaver-Burke and Eadie-Hofstee analysis facilitated by computer generated linear regression.

2.9 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE).

This was by the method of Laemmli [83] with 12.5% resolving gels (3.3% cross-linker). Fractions with low protein levels were concentrated by acetone precipitation. Gels were silver stained by the method of Wray[86] with variations of NH_4^+/Na^+ ratio to minimise the chances of protein being undetected.

2.10 UV Photoaffinity Labelling and Fluoroautoradiography.

Tissue fractions with high 11ß-HSD activity were diluted in buffer C to 0.05-0.25mg protein/ml (see text) and ^{3}H-steroid, 5mM NAD and 250mM DTT added to give final concentrations of 400µM NAD, 25mM DTT and 50nM ^{3}H-steroid. Unless otherwise stated

reactions were carried out in the wells of a 24-well plate (diameter 1.5cm) with the lid off and using a final reaction volume of 600µl. In the work leading to the purification of 11ß-HSD2 it was important to minimise UV damage to protein and reactions were at 37°C for a limited period (see Chapter 3 for details). For detecting 11ß-HSD2 protein in transfected mammalian cells the procedure was adjusted in this context to maximise labelling as minor damage to protein was less of a concern and accordingly labelling was carried out with 254nm UV light on supernatants cooled to 1-2°C and using at 0.25mg protein/ml. Transfected cells to be affinity labelled were homogenised, briefly centrifuged (1000rpm x 15s spin; to pellet lumpy debris) and the supernatant removed for labelling. Labelling reactions of placental 25000g pellet (at 0.15mg protein/ml) were run in parallel.

Labelled samples were acetone precipitated and resolved by SDS-PAGE[83], or 2-D electrophoresis. Finished gels, stained with Coomassie Blue to allow detection of major proteins and molecular weight standards, were processed for fluoroautoradiography in 'Entensify' solutions and vacuum dried before exposure to film.

2.11 2-D Electrophoresis.

2-D electrophoresis work involved running duplicate gels in parallel; usually one was silver stained (to visualise all the proteins) the other stained with Coomassie blue (to allow alignment between gels and to visualise SDS-PAGE protein standards) and processed for autoradiography. A set of 2-D electrophoresis protein standards was run under identical conditions on a third gel when new running parameters were used.

Conventional 2-D PAGE, i.e. isoelectric focusing (IEF) + SDS PAGE, used a variation of the method of O'Farrell[87]. NEPHGE 2-D electrophoresis (non-equilibrium pH gradient gel electrophoresis) conditions were based on the methods of O'Farrell[88] and Witzmann[89] with variations of sample buffer and rod gel composition. Rod-gels (17cm x 2.5mm: length x internal diameter) were cast from: 9M urea, 2% NP40, 4% acrylamide:bis (19:1), 3% pharmalytes (1.2% pH 6-8 + 1.8% pH 3-10), polymerised with 32µl 10% ammonium persulphate and 27µl TEMED per 20ml of rod gel mixture. Optimal results (higher yield, better resolution), especially for basic hydrophobic proteins, were obtained with a dodecyl maltoside based NEPHGE sample buffer:- 5.71g urea, 154mg dithiothreitol, 4ml 10% dodecyl maltoside, 750µl Pharmalytes (300µl pH 6-8, 450µl pH 3-10) 2.25 ml HPLC water adjusting pH to 4.2 (adding 90µl 5M HCl and 160µl HPLC water). This buffer, which was a great improvement, is close to solidifying at room temperature and is melted, warming to 30°C, to

adjust pH on preparation and on using. NEPHGE gels were run at 500V for 2250V.hours. After NEPHGE, rods were extruded into 10ml equilibration buffer for 7 min and either frozen or loaded directly onto second dimension SDS-PAGE gels. 150ml equilibration buffer consisted of 15ml 2-ß-mercaptoethanol, 9g SDS, 90ml HPLC water, 5.6ml 0.075% bromophenol blue and 37.5ml 4x Laemmli stacking buffer (0.4% SDS, 0.5M Tris-HCl pH 6.8). For both IEF and NEPHGE the second dimension was run according to the resolving gel system of Laemmli[83] using 12.5% gels (3.3% cross-linker). Silver staining was by the method of Wray[86].

Concentrated protein from preparative AMP affinity chromatography runs was divided among several 1.5mm thick preparative 2-D gels. Precautions were taken to reduce protein N-terminal modifications; the second dimension gel was pre-run in a Laemmli running buffer containing 1 part in 200 of freshly made 100mM glutathione and then changed to running buffer with 1 part in 1000 100mM sodium thioglycholate[92]. These precautions were successful in allowing N-terminal sequence to be obtained from other proteins in our laboratory.

2.12 Protein Blotting for Sequencing.

This was based on the CAPS (3[cyclohexylamino]-1-propanesulphonic acid) buffer method of Matsudera[93] and was performed from SDS-PAGE or second dimension gels on to PVDF (Problott) membranes using a Bio-Rad Mini Trans-Blot apparatus. Sections of gels to be blotted were washed for exactly 30 sec in CAPS transfer buffer (to allow shrinkage and removal of excess surface SDS). Electroblotting was for 75 min at 250 mA (90-110V). After transfer, the PVDF sheets were stained (amido black), destained (water), air dried and desired bands/spots cut out and stored at -70^{o}C.

2.13 Proteolytic Digests of PVDF Blotted Protein.

This was based on the method of Fernandez[94]. Membranes were cut into small pieces, blocked (0.2% PVP-40 in methanol for 45 min) and extensively washed in deionised HPLC water. Auto-digestion resistant trypsin was added in AHT buffer (20% **a**cetonitrile, 1% **h**ydrogenated Triton X100 in 100mM **T**ris pH8) to a concentration of 1:20 (trypsin : blotted protein) and digestion continued for 24 h at 37^{o}C. Fragments were eluted using vigorous sonication and washes (AHT buffer, then 0.1% TFA)[94]. Eluted peptides were concentrated,

removing acetonitrile, and loaded as a 100µl sample onto an Applied Biosystems microbore HPLC system fitted with a C8 RP300 column (1x250mm, 7µm particle size) equilibrated in solvent A (0.1% TFA/water). This was developed with a linear gradient of 0-100% solvent B (0.08% TFA in 80% acetonitrile/water) over 45min at 200µl/min. The spectrophotometric absorbence at 220nm (A_{220}) of eluate allowed collection of peptide peaks. Control digests (trypsin with PVP-40 blocked blank PVDF slices) were run in parallel to ensure identification of all peaks not due to the blotted protein substrate. A large peptide peak yielding no sequence which was thought to be the blocked N-terminal peptide was sub-digested in solution (50mM sodium phosphate pH7.8) with *Staph. Aureus* V8 protease at an estimated ratio of 20:1 (peptide/protease). Products were resolved on HPLC, as were those from a control (protease only) digest.

2.14 Amino Acid Sequencing.

Peptide peaks selected for sequencing were pyridyl-ethylated[101], to allow detection of cysteine residues, and loaded into an Applied Biosystems 477A automated amino acid microsequencer.

2.15 Degenerate Primer PCR and Cloning of 11ß-HSD2 cDNA Fragment.

Tissue was snap frozen and RNA extracted, as described[85]. RNA was treated with DNAse I, re-extracted to remove any contaminating genomic DNA and reverse transcribed using Promega Reverse Transcription system (#A3500) according to the manufacturer's instructions. Inosine-containing degenerate primers were designed based on the amino acid sequence of 5 11ß-HSD2 tryptic peptides; A, B, B2, C and D (t = top strand, b = bottom strand)(see Chapter 6). The primers from peptides B and C, generated the most useful results and had the following sequences (I = inosine):-

Bt = 5'-CA(A/G)GA(C/T)GCIGCICA(A/G)GA(T/C)CCIAA-3',
Bb = 5'-A(A/G)(A/G)TTIGG(A/G)TC(C/T)TGIGCIGC(A/G)TC-3',
Ct = 5'-(A/T)(C/G)ICCIGCIGGI(A/G)(A/C)IATGCCITA-3',
Cb = 5'-AGCICCIA(A/G)IIIIGG(A/G)TAIGGCAT-3'.

Initial screening, used the following protocol. 5µl of human placental cDNA, was heated, under mineral oil, at 96°C for 10 min and placed on ice. The reaction mixture was then added in a volume of 45µl to give a final reaction mix containing 70pmol of each degenerate primer, 50µmol dNTP, 2U TAQ polymerase in 1xPCR buffer. Reactions were

placed into a thermal cycler (HB-TR1 :Hybaid, Teddington, U.K.) with the block held at 90°C and the programme commenced: 5 cycles of [60s x 95°C, 45 s x 43°C, (43 55°C) x 1°C/3s, 90 s x 72°C] followed by 35 cycles of [60s x 95°C, 60 s x 50°C, 90 s x 72°C] and finally 10 min x 72°C. Subsequently, a specific DNA product (of 531bp) was efficiently amplified using the CtBb primer pair and the following programme: 5 cycles of [60s x 95°C, 60s x 47°C, (47 50°C) x 1°C/5s, (50 55°C) x 1°C/2s, 150 s x 72°C] followed by 40 cycles of [60s x 95°C, 60s x 50°C, (50 55°C) x 1°C/s, 150 s x 72°C] and finally 10 min x 72°C. This allowed the 531bp product to be directly cloned into pCRII (TA Cloning system v2, Invitrogen, San Diego, CA: #K2000-01) to yield clone pCRIICtBb.

2.16 PCR Screening of a pcDNA1 Human Placental cDNA Library.

Specific PCR primers nested within this 531bp fragment were designed (top primer, SCt, = 5'-ATCCGTGCTTGGGGGCCTATGGAACCT-3'; bottom primer, SBb = 5'-CTGCAGTGCTCGAGGCAGACAGTGACT-3'). These produced a strong band of the expected size (455bp) as the only product amplified by RT-PCR of human placental RNA. A human placental cDNA library in pcDNA1 (Invitrogen #A900-11) was then screened by increasing dilutions using this 455bp product to detect positive pools and adapting a PCR screening method[103] for use with plasmid libraries. Other specific primers were used to cross-check positives. PCR with one primer to the pcDNA1 vector arm and the other to the insert was used to estimate clone insert size. A partial 11ß-HSD2 clone was isolated (1177bp insert). On re-screening no clones long enough to be full length (>1.7kb: coding region (40k protein) + 3'UTR 1.7kb) were found that produced 11ß-HSD activity on expression in CHO cells.

2.17 Screening of a DR2 cDNA Library.

A DR2 human placental cDNA library (Clontech, Palo Alto CA: #HL1144x), with a higher proportion of longer cDNA inserts, was screened by conventional means[171], using the incomplete 11ß-HSD2 sequence isolated from the pcDNA1 library. Briefly, 700000 plaques were plated, duplicate filter lifts made, denatured, fixed, rinsed, dried and UV cross-linked before hybridisation in SSC/formamide buffer (6xSSC, 50% formamide, 5x Denhardt's solution, 0.5% SDS, 100µg/ml salmon sperm DNA) with random primed ^{32}P-dCTP labelled probe from the incomplete 11ß-HSD2 sequence. Washes were SSC/SDS based, finishing with one 15 min wash at 65°C in 0.2 x SSC/0.1%SDS. Positives from the primary screen were purified by a secondary screen at low density.

Secondary positives were converted from phage (DR2) to plasmid (pDR2) clones by means of the endogenous CRE/LOX recombinase of the host bacterial strain (*E.coli* AM1)[112] and were tested for 11ß-HSD2 enzyme activity by transfection into CHO cells, as described below. Nucleotide sequences were determined following sequencing of both strands by the dideoxy termination method using Sequenase v2 (Amersham/USB).

2.18 Nucleic Acid and Protein Sequence Analysis.

The 11ß-HSD2 cDNA sequence and predicted 11ß-HSD2 protein sequence were analysed using the computing facilities at the HGMP resource centre (Cambridge, U.K. [104]), specifically PredictProtein[105] and the GCG package[106]. Protein secondary structure was predicted by three methods, according to:- Chou-Fasman as amended for overall structure probability[74,107], Garnier-Osguthorpe-Robson[108], and Rost-Sander[105]. The features described are for regions where there was no disagreement in predictions (fig.7.2(b) also extends illustration to areas (grey regions in fig.7.2(b)) with a complete concordance between two predictions and where the third 'dissenting' prediction is not in complete agreement). Assessment of hydrophobicity was based on the Kyte-Doolittle index[109] while prediction of flexible and exposed/buried regions was according to the methods of Karplus-Schulz[73] and Emini[110], respectively. Estimates of percent identity in nucleic acid and protein alignments used the GAP algorithm[106] with standard settings of gap weight = 3, length weight = 0.1 for protein alignment.

2.19 *In Vitro* Translation.

The 11ß-HSD2 cDNA was subcloned into pGEM-11zf oriented so that the 5' end of the cDNA was adjacent to the vector T7 promoter. *In vitro* translation was performed with a T7 polymerase driven, rabbit reticulocyte based, coupled transcription/translation system (TNT® lysates: Promega #L4610), to which a methionine deficient amino acid mixture and ^{35}S-methionine were added. In a standard volume of 25µl, 0.5µg of subcloned 11ß-HSD2 plasmid DNA was added. Microsomal co-translational processing was examined by including 0-4.5µl of micrococcal nuclease-treated canine pancreatic microsomes in the standard 25µl incubation at 30°C for 90min. Control reactions to verify signal peptide cleavage (0.1µg *E.coli* ß-lactamase mRNA) and glycosylation (0.1µg *S.cerevisiae* -factor mRNA) activities of the microsomes were carried out in parallel. For autoradiography, 0.05-5µl of each reaction was run per lane on SDS-PAGE, stained with Coomassie blue, processed in Entensify fluoroautoradiography solutions (NEN/DuPont, Stevenage, U.K.) dried and exposed to Kodak X-OMAT AR film.

2.20 CHO cell Transfection.

CHO cells were maintained in Dulbecco's modified Eagle Medium, supplemented with 15% fetal calf serum, 100 IU/ml penicillin, 100µg/ml streptomycin and 200mM glutamine. 24h prior to transfection cells were seeded onto dishes at a density of $2x10^6$ cells/10cm plates. For transfection 5µg DNA (in 800µl Optimem) was mixed with 42µl Lipofectin (in 800µl Optimem) and incubated for 15 min at room temperature. Optimem was added to 10ml and the mixture was gently overlaid onto cells which had been washed in Optimem. After 24h the Optimem/DNA mix was removed and replaced with normal medium. Cells were harvested 24h later. The human placental 11ß-HSD2 cDNA was transfected into cells using the clone (in pDR2) isolated from library screening and there were appropriate controls for transfection efficiency. Assays of 11ß-HSD activity were either with intact cells (^{3}H-steroid added to the medium within the last 24h, as described[69]) or using homogenates of scraped cells (as below).

2.21 Kinetic and Inhibitor Studies on Transfected Mammalian Cells.

CHO cells were scraped, homogenised ($2x10^6$ cells/0.5 ml Buffer C: 0.02M Tris pH 7.7, 10% glycerol, 1mM EDTA, 300mM NaCl) centrifuged briefly (1000rpm x 15s, Eppendorf centrifuge; to pellet heavy debris), and the supernatant assayed for protein concentration and 11ß-HSD2 activity (as described in Chapter 2). 11ß-HSD2 assays contained 400µM NAD, unless otherwise stated, and were analysed by HPLC when the ^{3}H-steroid concentration was >2.5nM, TLC based analysis was also used at ^{3}H-steroid concentrations <5nM. Reaction products were identified (HPLC and TLC) by comparison with steroid standards run in parallel. Incubations were for 60min (120min for dexamethasone). Kinetic parameters were calculated from the initial velocity determinations, obtained with experiments performed with a wide range of substrate concentrations (0.3, 0.4, 0.8, 1.5, 2, 3, 4, 8, 15, 20, 40, 80, and 150nM ^{3}H-steroid, with 80 and 150nM omitted for corticosterone). Enzyme concentrations giving less than 30% conversion were used. Control ('vector-only' transfected cells) and blank assays were carried out in parallel.

2.22 Northern Hybridisation.

Adult human tissue samples were obtained at surgery, frozen within 10 min, and stored at -80°C. Most samples were normal tissue removed adjacent to a tumour on resection, this was the case for:- kidney (with adjacent adrenal), parotid, colon (splenic flexure), breast (with adjacent skin and dermis), stomach (with distal oesophagus). Normal ovary (pre-menopausal) was obtained at hysterectomy. Pancreas was an unaffected area in a pancreatic tail resected for chronic pancreatitis. Liver was from a partial hepatectomy in a young woman to remove a hepatic cyst (benign). Placenta was from a normal delivery. Regions of a normal human post-mortem (36h) brain were also dissected. RNA was extracted as described[85], separated on denaturing agarose/formaldehyde gels[171] (10μg/lane) and blotted onto Hybond N membrane (Amersham). A human multiple tissue northern blot was purchased from Clontech (#7756-1). 2μg of highly purified poly A RNA from fetal tissues (brain, lung, liver(female) and kidney) recovered following spontaneous abortions (at least 2 specimens pooled for each organ) had been run in each lane and blotted. The exact age ranges were:- fetal brain 21-26weeks, fetal lung 22-23 weeks, fetal liver 22-26weeks and fetal kidney 19-23weeks. Blots were hybridised with random primed ^{32}P-labelled p11ß2 AscI-DraI fragment (bases 217-1737 fig.7.2.) at 55°C overnight in hybridisation buffer (0.2M sodium dihydrogen phosphate, 0.6M disodium hydrogen phosphate, 5mm EDTA, 6% SDS and 100μg/ml denatured herring testis DNA). Washes were SDS/SSC based finishing with 0.1xSSC, 0.1%SDS at 65°C followed by autoradiography (-70°C, 3-8 days).

2.23 *In Situ* Hybridisation.

Cryostat sections (10μM) were cut from frozen samples of human kidney (normal tissue from the opposite pole to a discrete renal cell carcinoma) obtained at surgery and normal placenta. Sections were mounted onto gelatin, and poly-L-lysine, coated slides, and stored at -80°C. Slides were postfixed in 4% paraformaldehyde, washed in 2xSSC and incubated with pre-hybridisation buffer for 3h at 50°C, as previously described[10], before hybridisation with SP6 transcribed ^{35}S-UTP labelled antisense cRNA probes from XbaI linearized pCRIICtBb (531 bp of p11ß2, bases 654-1184 : dashed box in fig.7.2). Sense controls used T7 transcribed cRNA primed from HindIII linearized pCRIICtBb. RNA probes were denatured, added at a final concentration of $10x10^6$ cpm/ml in hybridisation buffer, applied to slides, as described[10] and incubated overnight at 50°C. Following hybridisation, sections were rinsed twice in 2xSSC, treated with RNase A (30 μg/ml, 60min, 37°C), and washed to a maximum stringency of 0.1xSSC at 60°C for 60min. After dehydration in increasing concentrations of ethanol, slides were exposed to autoradiographic

film (Hyperfilm $B_{max.}$). Slides were dipped in photographic emulsion (NTB-2, Kodak, U.K.), exposed in a light-tight box for 5 weeks, before being developed (D19; Ilford U.K.) and counterstained with 1% pyronin.

2.24 Raising Antisera and Western blotting.

Solid phase synthesis of an 11ß-HSD2 peptide HCLPRALQPGQPGT (residues 370-383, fig.7.2) with high predicted antigenicity was carried out. The peptide was N-terminally coupled to keyhole limpet haemocyanin and rabbits were inoculated with the conjugate and Freund's adjuvant, boosting monthly. Antiserum highly specific for 11ß-HSD2 and reacting to the peptide 370-383 were obtained after the fourth boost in one rabbit. Western blots were performed with the 11ß-HSD2 antiserum, at 1/10000 dilution (as primary antibody), using the Amersham ECL system and methods recommended in the manufacturers' protocols accompanying the ECL system reagents.

2.25 Immunohistochemistry.

Sections of human tissue, stored at -80°C, were thawed into neutral formalin fixative (for 8 h), paraffin processed and 4µm sections cut. After drying, sections were dewaxed, hydrated, and treated in 3% H_2O_2 (20 min) and blocked with 20% sheep serum in TBS (Tris buffered saline pH 7.6 with 1% bovine serum albumin and 0.1% sodium azide). This diluent was also used for primary and secondary antibodies. Immunostaining, using an avidin-biotin complex (ABC) method at room temperature, was as follows:- primary antibody (1/2000), 30min; TBS wash; secondary antibody (1/400 biotinylated sheep anti-rabbit $F(Ab)_2$ fragments, Boehringer Mannheim, Lewes, U.K.), 30min; TBS wash; Vectastain® ABC *Elite* reagent, 30min; DAB substrate/chromagen reagent, 5 min. Sections were counterstained in Mayer's Haematoxylin, dehydrated, cleared and mounted. Primary antibody preparations used were (a)11ß-HSD2 antiserum, (b) pre-immune serum from the same rabbit and (c)11ß-HSD2 antiserum pre-absorbed for 16h with peptide 370-383 at 50µg per ml of diluted antibody. Both (b) and (c) were used as controls for (a).

Chapter 3 : Solubilisation and Partial Purification of Human Placental 11ß-HSD.

3.1 Introduction.

Placental 11ß-HSD was clearly of great interest and seemed to have characteristics distinct from the 'liver-type' 11ß-HSD activity (see Chapter 4). In particular placental 11ß-HSD appeared to be NAD-dependent. In order to clarify whether it represented a different isoform and to allow study in detail it was essential to obtain tools (purified enzyme, specific antibody, encoding cDNA etc.) allowing its specific detection. To begin to pursue these goals it was essential to isolate the enzyme responsible for this 11ß-HSD activity in placenta as far as possible. There are cytosolic as well as membrane bound steroid dehydrogenases/reductases and the majority of successful purifications have been for the enzymes in the soluble group[188-190]. Although quite a number of the membrane bound enzymes have remained intractable to extensive purification, often despite extensive efforts (e.g. 5α-reductase where only 8-40 fold purifications have been reported [186,187]) a number have been purified to homogeneity, notably 'liver-type' 11ß-HSD[35]. One of the main reasons for this difference is that membrane-bound enzymes may require membrane components for full activity whilst even those which can be fully solubilised may be prone to aggregation and precipitation out of solution if they are brought into close proximity with chromatography matrices. Accordingly, whilst soluble enzymes are often successfully purified in active form by a procedure with several chromatography stages, this is very rare for membrane proteins. If membrane bound enzymes are successfully purified, this is often by using a very high resolution technique (typically affinity or immunoaffinity chromatography) in a short, one or two stage, purification procedure. This was the case for 'liver-type' 11ß-HSD where purification to homogeneity was achieved with NADP-agarose[35].

Thus, in the purification of human placental 11ß-HSD the strategy was to use subcellular fractionation and, whether the enzyme was cytosolic or membrane bound, to screen for an affinity chromatography matrix providing useful purification. As the placental enzyme metabolised glucocorticoids, and appeared to prefer NAD as co-substrate, affinity matrices with NAD or glucocorticoids as ligands were to be tried.

3.2 Results.

3.2.1 Following placental 11ß-HSD activity.

In initial experiments on human placenta it soon was clear that [^{3}H]-corticosterone and [^{3}H]-cortisol produced qualitatively similar results, but [^{3}H]-corticosterone was clearly a better substrate for placental 11ß-HSD than [^{3}H]-cortisol (see Chapter 4). Moreover, separation of substrate and product on HPLC was better in assays with [^{3}H]-corticosterone. 11ß-dehydrogenase activity was the activity to be purified in these studies because 11ß-reductase in the human placental tissue studied (full term placentae) was only a very minor activity (less than 5% of 11ß- dehydrogenase at pH 7.5). Thus the fractionation and purification of the enzyme was to be followed by assaying its activity to metabolise ^{3}H-corticosterone to 11-dehydrocorticosterone.

3.2.2 Subcellular Fractionation.

Human placental 11ß-HSD activity was well preserved during subcellular fractionation. Placental 11ß-HSD was clearly a membrane-associated protein (as >95% activity remained in the membrane bound fraction, pelleting by 110000g) and was most abundant in the 25 000g pellet, which contained over two-thirds of the total enzyme activity in under one sixth of the total protein; five-fold higher than in the microsomal 110 000g pellet, (fig.3.1). Moreover, the 25 000g pellet had as high a specific activity as the 110 000g microsomal pellet, and its activity was stable and more rapidly prepared. Differential centrifugation of the 25 000g pellet did not produce any useful refinement. This fraction was used for further purification, and has been found to be very stable on storage at -70^{o}C, with no decline in activity in over 2 years. 11ß-HSD assays of this human placental 25000g fraction were at protein concentrations of 2-5µg/ml which gave percentage conversions of 10-40% (the desired range as defined in Materials and Methods).

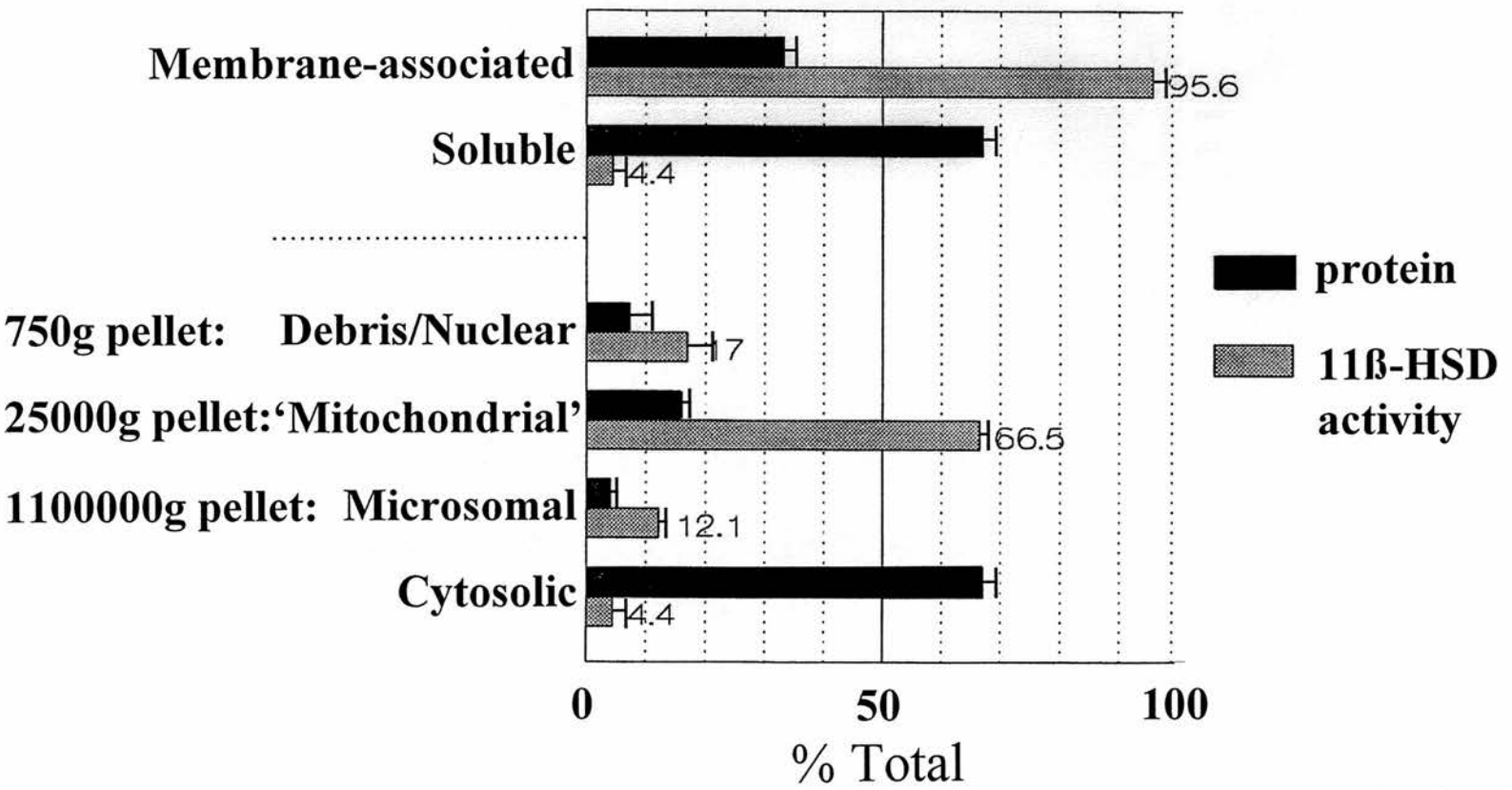


Fig 3.1 Distribution of 11ß-HSD activity amongst sub-cellular fractions of human placenta. The distribution of total protein and 11ß-HSD activity amongst the subcellular fractions generated from placental homogenates are shown. The 25000g pellet (mitochondria and heavy microsomes) contains two-thirds of the 11ß-HSD activity in under one sixth of the total protein. Bars represent mean (± SE, n=3) of total in whole tissue homogenate (the mean figure for 11ß-HSD activity is also given).

3.2.3 Solubilisation.

3.2.3.1 Increasing Ionic Strength.

The criteria for designating 11ß-HSD activity "soluble" is that it remains in the supernatant after centrifugation at 110 000g for 1 h. Increasing salt concentration is sufficient to solubilise a proportion of membrane proteins which are only associated with the membrane by surface contacts that dissociate with increased ionic strength[91]. Such solubilisation was attempted with placental 11ß-HSD (from resuspended 25 000g pellet) using a sodium chloride concentration ranging from 0 to 2.4M. The ionic strength clearly affected 11ß-HSD activity before centrifugation as shown below in Table 3.1.

Table 3.1 Effect of ionic strength on placental 11ß-HSD activity.

BUFFER IONIC STRENGTH (M)	RELATIVE 11ß-HSD ACTIVITY
0 M NaCl	100%
0.15M	148%
0.3M	159%
0.6M	111%
1.2M	59%
2.4M	6%

0.3 M NaCl produced the maximum increase (doubling) of soluble 11ß-HSD activity compared to no NaCl. However, this was from only 0.9% to 1.8% of 11ß-HSD activity and when corrected for the increased activity before centrifugation was only a 1.1-1.2% solubilisation. As 99% remained insoluble it was clear placental 11ß-HSD was an integral membrane protein rather than merely membrane surface associated and that detergent would be required for its solubilisation.

3.2.3.2 Detergent Solubilisation.

A screen was performed using 11 detergents of diverse classes[90], at concentrations of twice and half their critical micelle concentrations (CMC). Where the CMC was quoted as a range, half the lower limit and twice the mid-range were used. Following this preliminary screen,

conditions were refined for the most promising detergent (CHAPS), the related detergent Bigchap, and the detergent used to solubilise 11ß-HSD1 for purification (Triton DF18)[35]. The total of 13 detergents tested are given below in Table 3.2. along with their CMC and the concentrations used (mM).

Table 3.2-Detergents screened in solubilising human placental 11ß-HSD.

DETERGENT	CMC(mM)	CONCENTRATIONS USED(mM).
CHAPS	6-10	3 and 16, 1.5, 4, 5, 6, 7.5, 10
taurodeoxycholic acid	1-4	0.5 and 5
Polyoxyetylene-10-lauryl ether	0.20	0.1 and 0.4
digitonin	3.2-4	1.6 and 7.2
n-octylglucoside	20-25	10 and 45
Tween2	0.05-0.06	0.025 and 0.11
Tween 80	10-12,5	5 and 22
sodium dodecyl sulphate	7-10	3.5 and 17
n-lauryl sarcosine	13.7	6.8 and 27
Triton X100	0.2-0.30	0.1 and 0.5
zwittergent 3-10	24-40	12.5 and 65
Bigchap	2.9-3.4	0.7, 1.4, 2.8, 4.2, 6.3, 6.8
Triton DF18	unknown* ?≈0.15%	0.005%, 0.02%, 0.04%,0.08% 0.16%, 0.24% and 0.4%

* The molecular weight of this detergent was not available so concentration is expressed as %(v/v).

All solubilisation was carried out at 0-2°C. After 30 min, the mixture was centrifuged at 110 000g for 1 h. Supernatant containing soluble enzyme was carefully removed and the percentage of the pre-solubilisation 11ß-HSD activity remaining in this detergent solubilised fraction is shown for the detergents screened in fig,3.2. Placental 11ß-HSD activity was inactivated by concentrations of detergents required fully to transform membrane proteins into micelles (detergent concentration >> CMC), however a limited though useful solubilisation

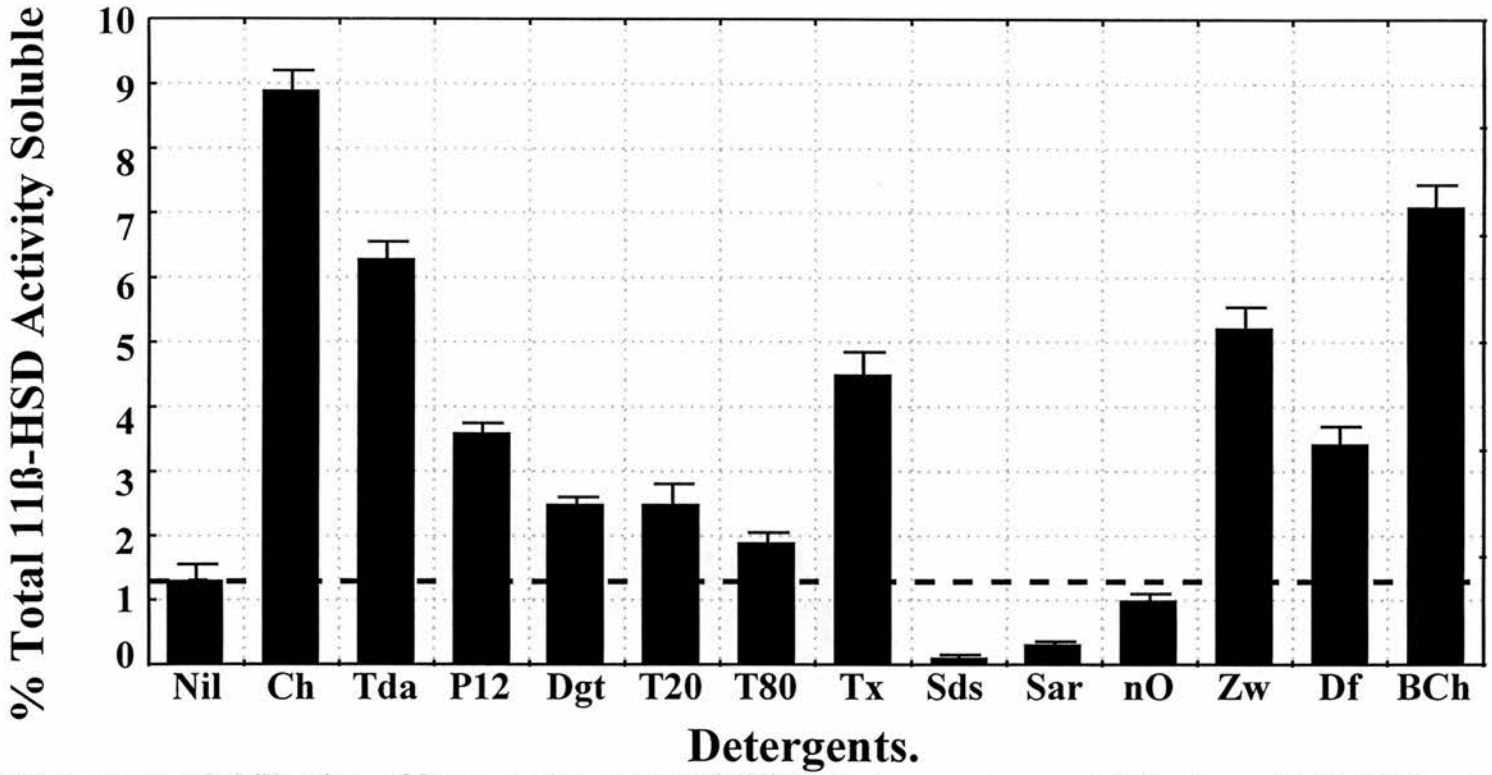


Fig 3.2 Detergent solubilisation of human placental 11ß-HSD. Best percentage solubilisation of 11ß-HSD activity from human placental 25000g pellet fraction acheived with screening concentrations of 13 detergents compared to activit remaining soluble with no detergent (dashed line). Results shown as mean±SE, n=4. The following abbreviations are used: Nil = no detergent, Ch = CHAPS, Tda = taurodeoxycholic acid, P12 = polyoxyelene-lauryl ether, Dgt = digitonin, T20 = Tween 20, T80 = Tween 80, Tx = TritonX100, Sds = sodium dodecyl sulphate, Sar = N-lauryl sarcosine, nO = n-Octylglucoside, Zw = Zwittergent3-10, Df = Triton DF18, BCh = Bigchap.

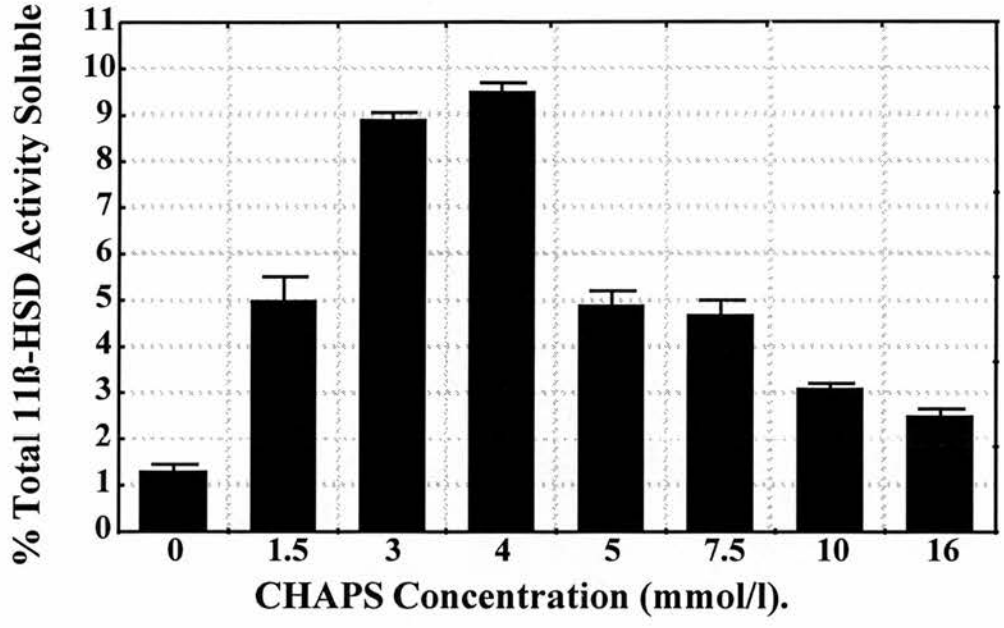


Fig 3.3 Refinement of human placental 11ß-HSD solubilisation with CHAPS. A range of concentrations of CHAPS (the most promising detergent) were screened revealing 4mmol/l was optimal for placental 11ß HSD solubilisation. (mean±SE, n=4).

was achieved with gentle conditions (detergent concentration < CMC), being best with zwitterionic detergents with bile acid or steroid-like head groups (such as CHAPS, Bigchap and taurodeoxycholic acid).

Following this preliminary screen, conditions were refined for the most promising detergent (CHAPS) as shown in fig.3.3, the related detergent Bigchap, and the detergent used to solubilise 11ß-HSD1 for purification (Triton DF-18) [35]. The best solubilisation achieved with Bigchap and Triton DF18 are shown in fig.3.2. 4mM CHAPS in combination with buffer C (which contains 0.3M NaCl) was found optimal, solubilising 40-45% of the 25000g pellet protein and 9.5% of its 11ß-HSD activity (CHAPS (9.5%) > Bigchap (7.1%) > taurodeoxycholic acid (6.3%) >Zwittergent3-10 >rest). This represented approximately a 7-8 fold increase over no detergent. Moreover on repeated re-extraction of the initially insoluble protein with 4mM CHAPS, 13-20% of the original 11ß-HSD activity could be solubilised.

3.2.4 Affinity Chromatography.

Since, 11ß-HSD uses NAD and glucocorticoids as substrates, columns with ligands mimicking these were screened. It was found that one matrix, N6-linked 5'-AMP-agarose (Sigma # A3019) bound substantial 11ß-HSD activity that could be eluted with cofactor (NAD>NADH>5'AMP>>NADP). Two other kinds of 5'-AMP-agarose (ribose hydroxyl (Sigma # A8895) and C8-linked (Sigma # A1271)) and two types of NAD-agarose were ineffective with a small percentage (2%) binding to the third type of NAD-agarose available (linked at C8, Sigma # N1008), eluting specifically with NAD. 11ß-HSD activity did not bind to NADP-agarose, cAMP-agarose or to dexamethasone-agarose (Sigma # D4657, 0.5-1µmol/ml dexamethasone immobilised at C17).

Preliminary studies with this N6-linked 5'AMP-agarose revealed it to be very useful for 11ß-HSD2 purification. Loading human placental CHAPS solubilised extracts (containing solubilised 11ß-HSD) onto this matrix, washing and using sequential elution with 1mM NAD, NADP and AMP, a large proportion of the human placental 11ß-HSD activity eluted specifically with NAD (fig.3.4(a)). Human placental 11ß-HSD fractions which eluted from the column were analysed by SDS-PAGE (fig.3.4(b)). A protein of M_r 40 000 was seen to co-

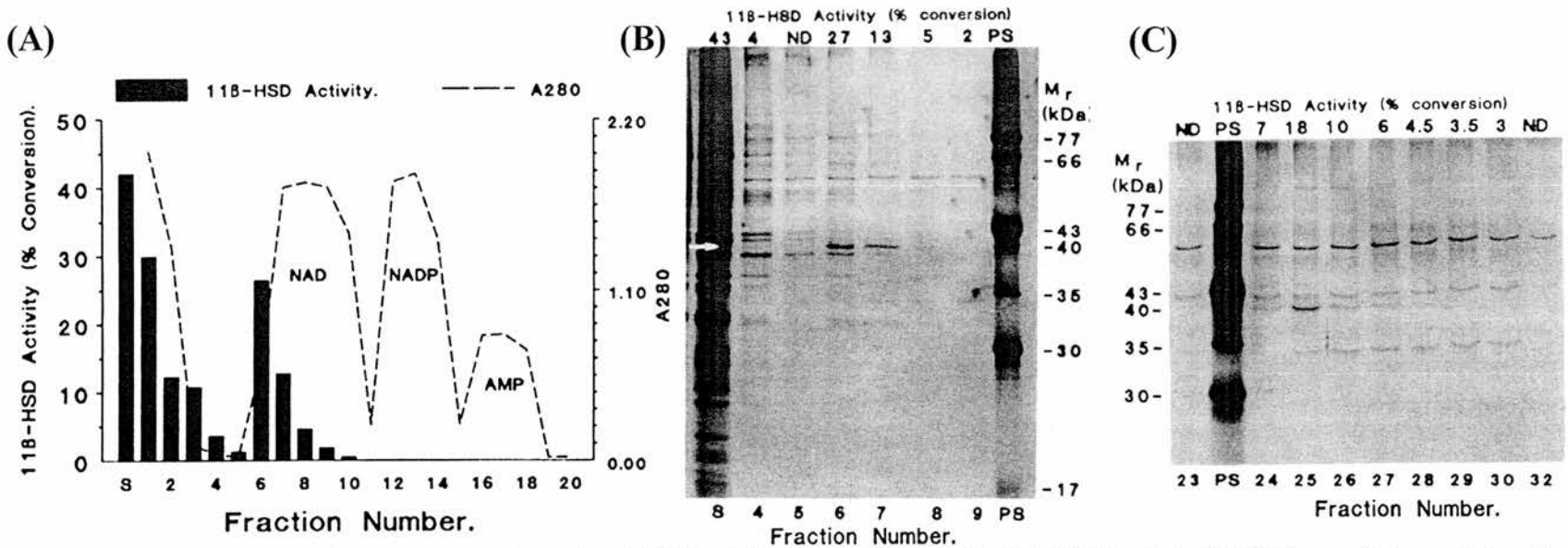


Fig 3.4. AMP-affinity chromatography of solubilised human placental 11ß-HSD. (A) 11ß-HSD activity of fractions assayed with corticosterone and 400µM NAD added. Spectrophotometric A_{280} demonstrating the falling protein concentration then showing cofactor addition. **(A).** Fraction S = detergent solubilised tissue extract loaded. Fractions: 1-2, flow through/wash; 3-5, wash; 6-20, alternating cofactor elutions (as indicated) and brief washes. **(B+C).** 12.5% SDS-PAGE gels (silver stained). **(B).** Gel of experiment shown in (A). Protein from 25µl fraction S and 500µl fractions 4-9 were loaded onto respective lanes. Corresponding 11ß-HSD activity (shown above gel) was assayed with corticosterone and 400µM NAD and used 15µl of each fraction. ND = not detectable. PS = Protein standards which included addition of 100ng malate dehydrogenase (M_r 35K). The protein which repeatedly segregates with 11ß-HSD activity migrates at the position arrowed(M_r 40K). The factor of purification of 11ß-HSD in lanes 4, 6, 7 and 8 compared to fraction S was 40-50, >1000, >1000 and >600 respectively. **(C).** Gel showing proteins and 11ß-HSD activity which eluted with NAD (beginning lane 24) after long wash. The 40K putative 11ß-HSD is the only protein consistently segregating with 11ß- HSD activity.

elute with 11ß-HSD activity (fig.3.4(b)). In multiple fractions over many chromatography experiments (under a variety of conditions) the 40K protein band, and no other, was seen to repeatedly segregate with 11ß-HSD activity (as shown in figs.3.4(b) and 3.4(c)). This contrasts with the rat liver 11ß-HSD isoform which has a M_r of 34 000 [35]. The quantity of protein in the fractions with 11ß-HSD activity was estimated by comparison with known quantities of protein standards run in parallel. The purification achieved was in excess of 1000-fold (fig.3.4(c)).

3.3 Discussion.

Thus, placental 11ß-HSD2 appears to be an integral membrane protein that requires factor(s) present in membrane subfractions of placenta which are not satisfactorily substituted by a wide range of detergents. Nonetheless a method allowing 13-20% of activity to be solubilised rapidly had been defined and this was sufficient to permit the purification to proceed further. It was obvious therefore that purification of this enzyme to homogeneity was going to be very difficult. Indeed, it was known that several groups, especially one in Chile collaborating with the late Dr Carl Monder (who had purified 11ß-HSD from rat liver) [191] had made extensive attempts to purify 11ß-HSD from human placenta but the difficulties in its solubilisation prevented any useful purifications. The group in Chile had found the best detergent screened to be Triton DF-18, but the solubilisation was poor and the purification was eventually abandoned. Several years later Lakshmi *et. al.*. (who had purified rat liver 11ß-HSD with Monder) reported a partial solubilisation of 11ß-HSD from human placenta with Triton TX100 but it appears this did not permit an extensive purification. In fact, a purification ≈10-fold is the only report this group made of this work [63]. Similarly, a group from Australia had attempted to purify NAD-dependent 11ß-HSD activity from rat or pig kidney and had been unable to solubilise it effectively and so the attempt at purification was abandoned[181].

As the work above defined a method allowing a modest solubilisation and purification of >1000-fold leading to identification of the likely protein responsible this represented a considerable advance on previous efforts. Other findings during this work and characterisation of the partially purified placental 11ß-HSD activity led to substantial evidence this isoform

was distinct from the 'liver-type' 11ß-HSD activity (see Chapter 4) and also gave insights into the likely kinetic mechanism of the placental 11ß-HSD enzyme (see Chapter 11).

Chapter 4 : Characterisation of Placental 11ß-HSD Clearly Identifies it as a New Isoform : 11ß-HSD Type 2.

4.1 Introduction.

As outlined in the Chapter 1, 11ß-HSD was purified from rat liver in 1988 [35] and an encoding cDNA cloned in 1989. However, within several tissues, particularly the distal nephron, there are striking discrepancies between the presence of 11ß-HSD enzyme activity, and the absence of the 'liver- type' protein (by immunohistochemistry) [15,17] or 'liver-type' (1.7 kb) mRNA[39,49]. This, and other evidence suggesting the presence of variant tissue-specific forms of 11ß-HSD[38,39,45,46,48], led to speculation about a second 11ß-HSD enzyme[167]. Work on renal DCT, particularly supported this view with an apparent NAD-dependent 11ß-HSD isoform detectable by histochemical activity staining[50,168].

As the methods developed in Chapter 3 had allowed production of substantially purified 11ß-HSD from human placenta it was possible to characterise this enzyme activity and compare and contrast it to the 'liver-type' 11ß-HSD from rat liver microsomes. To determine if differences observed were solely due to differences between species parallel investigations in the rat placenta were made when possible. Human placental 11ß-HSD was found to be most abundant in the 25 000g pellet (see Chapter 3). The same fraction was used for rat placenta. For rat liver, microsomes (110 000g pellet) were used as these were the source of the purified enzyme[35] for which the encoding gene has been cloned[36,46]. Finally, some comparison is made in the discussion with what was known of the 11ß-HSD activity derived from rabbit cortical collecting duct cells[64] which was published after completion of the work in this Chapter.

4.2 Results.

4.2.1 Tissue activities compared.

For all 3 tissues [^{3}H]-corticosterone and [^{3}H]-cortisol produced qualitatively similar results, but [^{3}H]-corticosterone was clearly a better substrate for 11ß-HSD than [^{3}H]-cortisol (as illustrated for human placenta in fig.4.1(a)). Moreover, separation of substrate and product on

HPLC was better in assays with [^{3}H]-corticosterone. Thus, corticosterone was mainly used in the studies presented below.

11ß-dehydrogenase activity was concentrated upon in these studies as 11ß-reductase in human and rat placental tissue (full term placentae) was only a very minor activity (0-5% of 11ß-dehydrogenase at pH 7.5). Moreover, placental 11ß-reductase was unstable and its activity underwent a progressive decline at 37^{o}C. This attenuation of activity was detectable even at 10 minutes. All studies compared 11ß-dehydrogenase in the 25 000g pellet of placental tissues with rat liver microsomal 11ß-HSD. Comparing the three tissues, rat placental 11ß-HSD was found to be the least abundant, most labile and was the only activity markedly attenuated by freezing and thawing. Accordingly rat placental fractions were freshly prepared and used rapidly.

4.2.2 11ß-HSD Cofactor Dependence.

To define and compare the cofactor preference of 11ß-HSD in the three tissues the activity of 11ß- HSD was determined when the concentration of NAD, or NADP, varied across the range 0-1000µM (fig.4.1). Both human and rat placental 11ß-HSD preferred NAD (ANOVA: $p<0.001$ for both tissues), whilst the rat liver enzyme preferred NADP ($p<0.001$). The activity with 100-1000µM cofactor was comparable in the three tissues (largely 30-45% conversion), whereas, with no cofactor, the activity was 22% conversion for rat liver and only 2% and 5% for human and rat placenta respectively. Although there appeared to be no NAD-dependent 11ß-HSD activity in liver microsomes, the high activity without addition of exogenous cofactor (which has been noted before in liver[169]) made this difficult to assess. Minor NADP-associated 11ß-HSD activity was seen in human and rat placental fractions. Eadie-Hofstee analysis of the data for human placental 11ß-HSD show rectilinear plots with NAD (apparent K_m for NAD = 13µM, V_{max} = 180 pmol/mg protein/min), but curved plots with NADP, suggesting the possibility that two distinct enzymes (with low and high K_m for NADP) contribute to the minor NADP-associated activity.

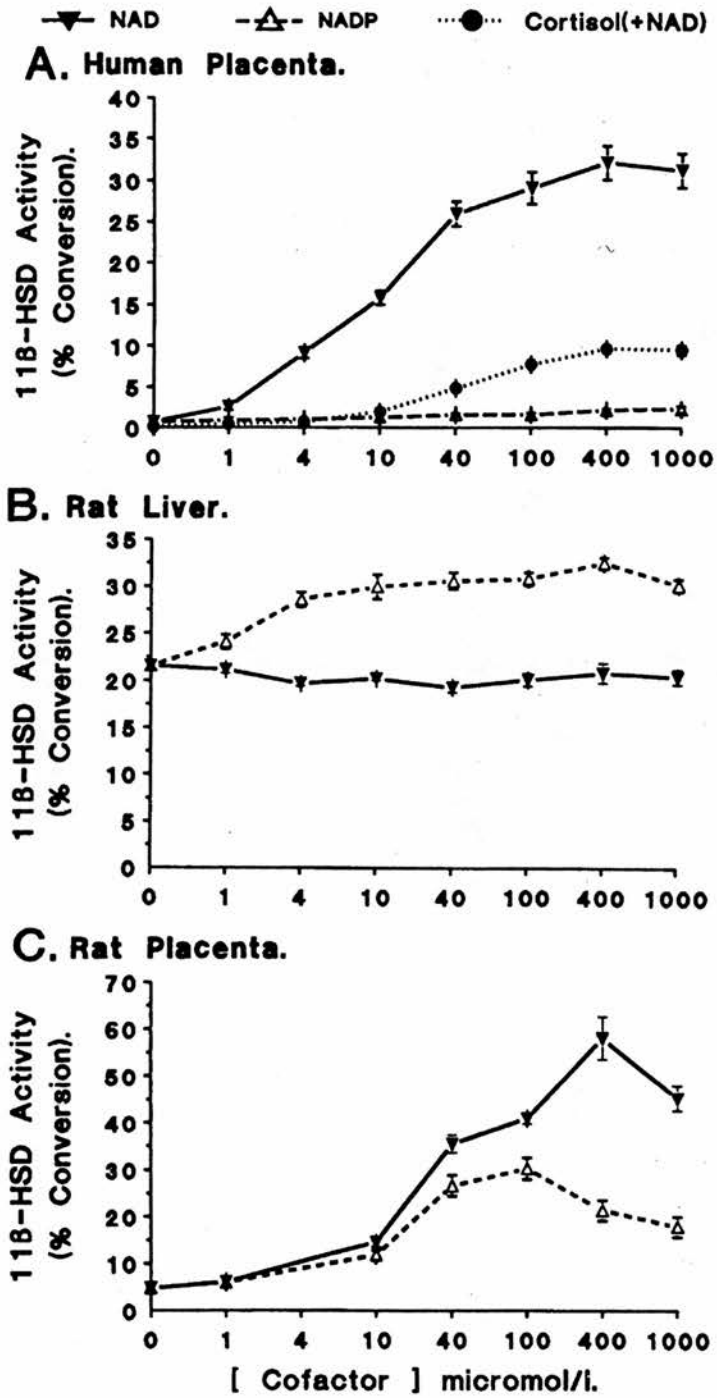


Fig.4.1 Cofactor dependence of 11ß-HSD activity. 11ß-HSD activity (mean±SE) in (A) human placenta, (B) rat liver and (C) rat placenta with corticosterone as substrate in the presence of 0-1000μM cofactor (NAD or NADP). Protein concentrations used in assays were:- human placenta, 2μg/ml; rat liver, 50μg/ml; rat placenta 500μg/ml. Human placental 11ß-HSD activity with cortisol and NAD shown too (with cortisol and NADP conversion remains <2%). Rat placenta n=4, otherwise n=5.

4.2.3 pH-dependence of 11ß-HSD activity.

The variation of 11ß-HSD activity in the 3 tissues over pH range 5-11 is shown in fig.4.2. The similarity between human and rat placental 11ß-HSD activity with either NAD or NADP is striking, as is their difference from 11ß-HSD activity in rat liver microsomes. Placental 11ß-HSD has a broad pH optimum of 7-8.5 with NAD (peak 7.5-8 in human placenta). With NADP as cofactor the optimum is more alkaline (pH 8-9). Above pH 9 placental 11ß-HSD activity falls off sharply (especially NAD-associated activity). In contrast, pH 9-10 is the optimum for rat liver 11ß-HSD. The activity of liver 11ß-HSD is largely or wholly independent of added NAD (fig.4.1(b)) . The liver 11ß- HSD activity with added NAD may be due to a relatively high level of endogenous cofactor[170]. Reductase activities for all 3 tissues were optimal at more acidic pH: pH5 for rat liver 11ß-reductase with NADPH and pH 5-6 for human placental reductase. Solubilised 11ß-HSD pH profiles are discussed below.

4.2.4 Solubilisation of 11ß-HSD Activities.

Detergents were used to solubilise 11ß-HSD, which was membrane associated in all 3 tissues. 4mM CHAPS was found to be optimal for solubilising human placental 11ß-HSD (see Chapter 3). This is a lower concentration than used in other studies on 11ß-HSD[35,37]. Triton X100 has been shown to be a good detergent for solubilisation of rat liver[30] 11ß-HSD. 0.06% Triton X100 was optimal for solubilising rat liver microsomal 11ß-HSD activity. This corresponds to a detergent/protein ratio of 0.2 - the optimum found by Monder and Lakshmi[30].

To compare detergent solubility, all 3 tissues were treated with (i)0.06% Triton X100, (ii)4mM CHAPS or (iii) buffer only. For each detergent the proportion of total protein solubilised was similar for all 3 tissues (around 65% with Triton X100, 45% with CHAPS). 11ß-HSD assays were performed on samples taken (i) before centrifugation and from the detergent-solubilised fraction (110 000g supernatant) at (ii)1 hour and (iii)24hours after centrifugation. Results of (i) and (ii) are shown in fig.4.3. Solubilisation of rat liver 11ß-HSD was very efficient compared to placental 11ß- HSD. Addition of either Triton X100 or CHAPS increased rat liver 11ß-HSD activity (before centrifugation) demonstrating considerable latency. Following

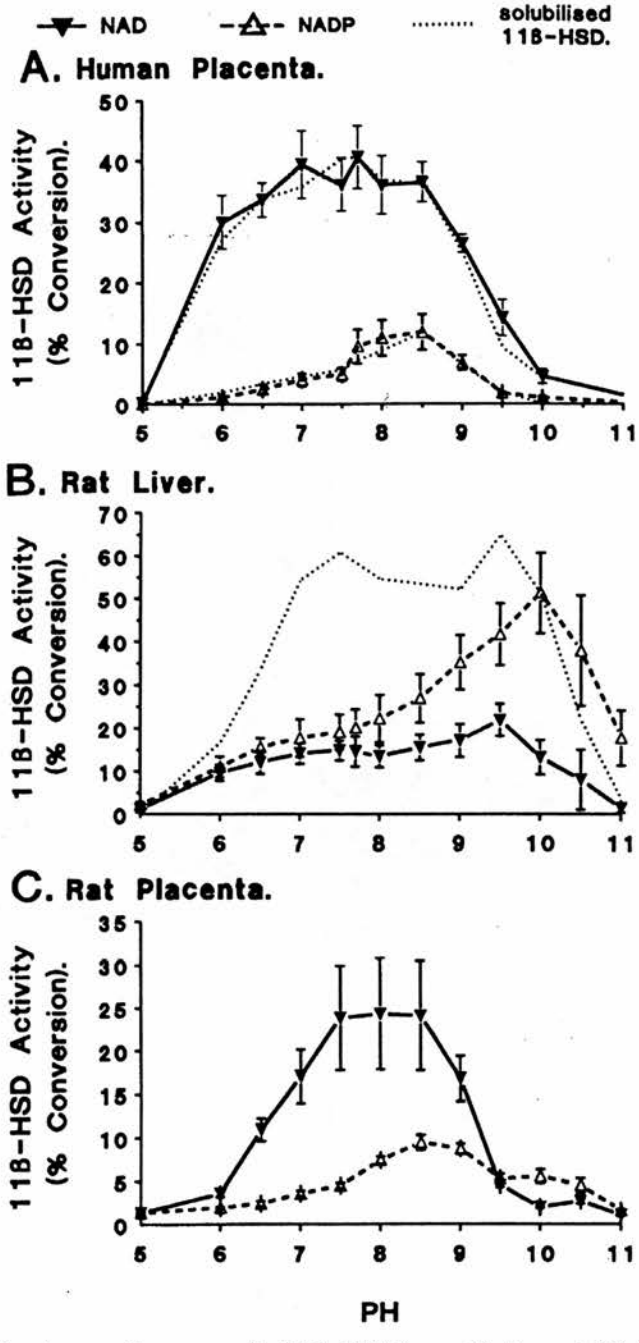


Fig.4.2 pH dependence of 11ß-HSD activity. 11ß-HSD activity (mean±SE) in (A) human placenta, (B) rat liver and (C) rat placenta with corticosterone and 400µM NAD, or NADP, across pH range 5-11. Protein concentrations used in assays were:- human placenta, 2µg/ml; rat liver, 20µg/ml; rat placenta, 100µg/ml. Dotted line shows pH profile of solubilised enzyme (with 0.06% Triton X100 or 4mM CHAPS for rat liver and 4mM CHAPS for human placenta). These are superimposed (to allow comparison of shape) to intersect corresponding graph without detergent at its pH optimum (human placenta NAD pH 7.7,NADP pH 8.5; rat liver NADP pH 10). Rat placenta n=4, otherwize n=5.

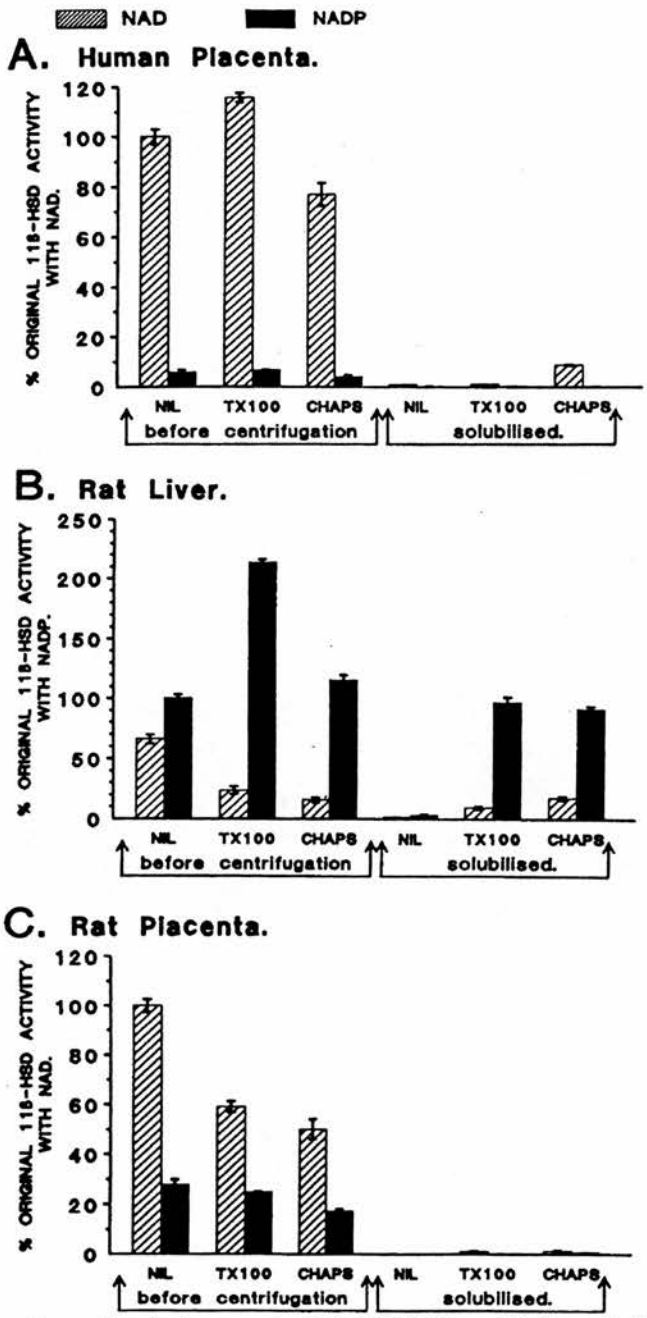


Fig.4.3 Behaviour in detergent of 11ß-HSD activity. Activity of 11ß-HSD (mean±SE) in (A) human placenta, (B) rat liver or (C)rat placenta with corticosterone and 400μM NAD or NADP. All tissues were suspended at 3mg/ml in:- buffer C only (Nil); buffer C + 0.06% Triton X100 (TX100) or buffer C + 4mM CHAPS and assayed firstly, before centrifugation and secondly in supernatant after centrifugation at 110 000g for 60 min. Results are expressed as percentage of activity with preferred cofactor, without detergent and before centrifugation, with protein concentration as follows:- human placental, 2μg/ml; rat liver, 20μg/ml and rat placenta 300μg/ml. Rat placenta n=4, otherwise n=10.

centrifugation a large proportion of the activity was soluble: between 90 and 100% of that before detergent (compared to <2% when no detergent was added).

In contrast, human placental 11ß-HSD showed minimal latency and was sparingly soluble (9%) in CHAPS, whereas, the use of Triton X100 did not significantly increase 11ß-HSD activity solubilised (1±0.08% compared to 0.84±0.02% without detergent). Rat placental 11ß-HSD behaved similarly to human placental 11ß-HSD (compare fig.4.3(a) with 4.3(c)), however, analysis of rat placental 11ß-HSD was hindered by the lability of the enzyme. The soluble 11ß-HSD activities in both human placenta and rat liver extracts were stable with >80% remaining after 24 hours at 0-2°C. Solubilisation changed the pH profile of rat liver 11ß-HSD, whereas very little change occurred when the human placental enzyme was solubilised. The altered liver 11ß-HSD pH profile remained very different from those of the placental enzymes in the pH 8.5-10 range (fig.4.2).

Thus, using the same detergent and protein concentrations, the liver and placental 11ß-HSD enzyme activities behaved very differently.

4.2.5 Comparative Affinity Chromatography.

Human placental and rat liver CHAPS solubilised extracts (containing solubilised 11ß-HSD) were subjected to affinity chromatography using columns of the N6 AMP-agarose matrix identified in Chapter 3 as achieving 1000-fold purification of placental 11ß-HSD. Sequential elution was with 1mM NAD, NADP and AMP (for human placenta:figs.3.4(a) and 4.4(a)) with NADPH following this for rat liver. A large proportion of the human placental 11ß-HSD activity eluted specifically with NAD. By contrast, liver 11ß- HSD had no affinity for the same column and no 11ß-HSD activity eluted with any of the pyridine nucleotides (fig.4.4(b)). When the human placental 11ß-HSD fractions which eluted from the column were analysed by SDS-PAGE a protein of M_r 40 000 was seen to co-elute with 11ß-HSD activity (Chapter 3, fig.3.4). This contrasts with the rat liver 11ß-HSD isoform which has a M_r of 34 000 determined by SDS-PAGE[35]. This suggested that the placental isoform was NAD dependent and distinct from the known isoform cloned from rat liver.

Samples of rat kidney were processed and run, in a similar fashion, on affinity chromatography in order to see if this placental isoform was likely to be similar to the isoform of 11ß-HSD

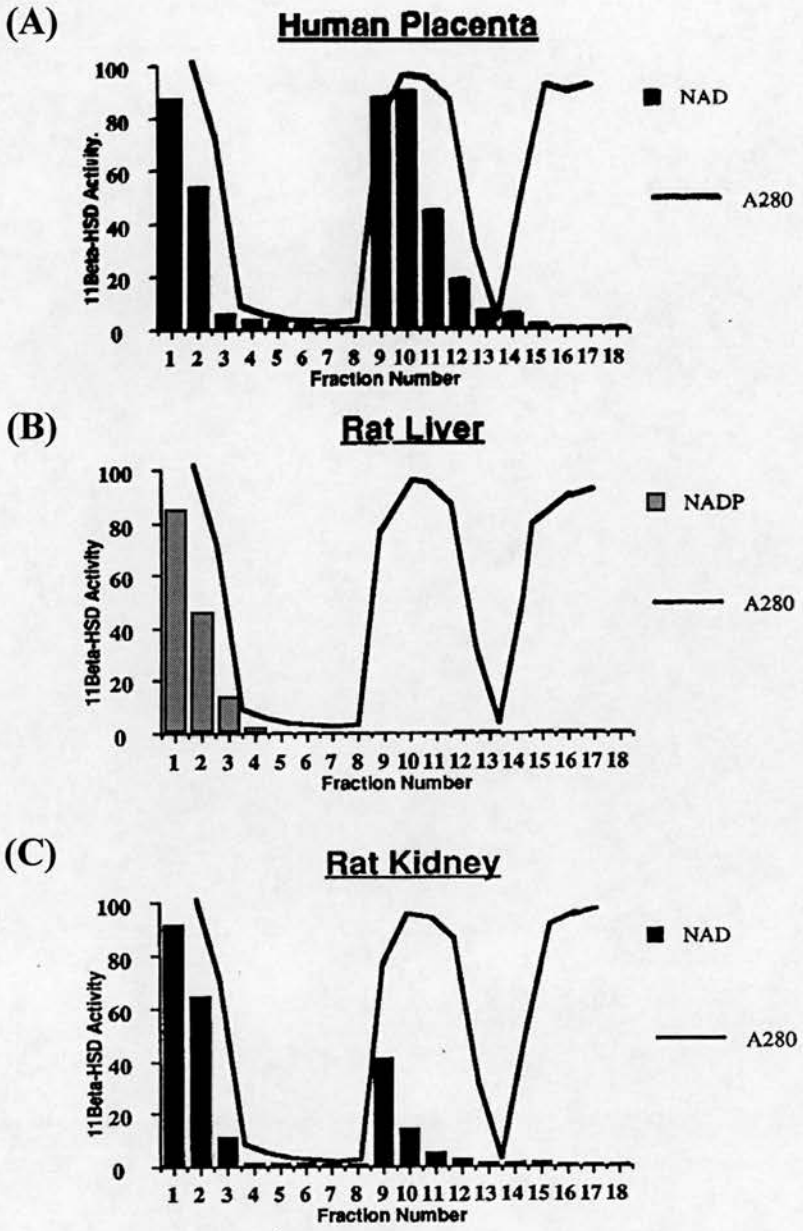


Fig.4.4 Comparative Affinity Chromatography for 11ß-HSD. AMP affinity chromatography of CHAPS solubilised 25000g fractions from (A) human placenta and (C) rat kidney; assaying fractions for NAD-dependent 11ß-HSD activity. Both clearly show specific binding of 11ß-HSD2-like activity. In contrast, panel (B) shows chromatography of CHAPS solubilised rat liver microsomes revealing that the 11ß-HSD activity in this tissue (which is NADP-dependent) has no affinity for the same AMP affinity matrix. For all three tissues the solubilised tissue extract was loaded on to the columns which were then: washed (fractions 1-8), eluted with 1mM NAD(fractions 9-12), washed (fraction 13) and finally eluted with 1mM NADP (beginning during fraction 14).

supposed to be present in distal nephron. As with human placenta and unlike rat liver, a significant quantity of 11ß-HSD activity bound specifically to the column and eluted with NAD(fig.4.4(c)). This provided evidence that an isoform similar to placental 11ß-HSD was indeed present in kidney[180].

4.2.6 Assessment of substrate and cofactor preferences of partially purified human placental 11ß-HSD.

CHAPS produced competitive inhibition of human placental 11ß-HSD, reaching 50% (K_i) at 0.37mM. Estimates of kinetic parameters for the partially purified enzyme eluted both with and without CHAPS were in good agreement when corrected for this competitive inhibition. Thus, the K_m of the partially purified human placental 11ß-HSD was 14±1 nM (mean ± SE) for corticosterone and 54±14 nM for cortisol, with a V_{max} of approximately 25nM[mg protein, min]$^{-1}$ with either steroid. The cofactor preference of placental 11ß-HSD was examined by removal of NAD by repeated filtration in a Centricon-10 apparatus (see Chapter 2). At a residual NAD concentration of below 2µM, addition of 400µM NADP promoted an increase in 11ß-HSD activity. When residual NAD was above 2µM, addition of 400µM NADP was without effect. Thus a >200-fold excess of NADP over NAD was required to demonstrate an effect on the enzyme.

When gradients of nucleotide cofactors were used to elute bound human placental 11ß-HSD from the AMP affinity column the 11ß-HSD activity began to elute at thresholds of 30µM NAD and 300µM AMP but did not elute at all during a 0-3000µM NADP gradient (fig.4.5). After the AMP gradient a small amount of enzyme eluted with 3mM NAD. After the NADP gradient a large amount of 11ß-HSD activity eluted with NAD (fig.4.5). This again clearly demonstrated the NAD-dependent nature of human placental 11ß-HSD enzyme which was in contrast to the NADP dependent 'liver-type' 11ß-HSD isoform exemplified by the activity in rat liver.

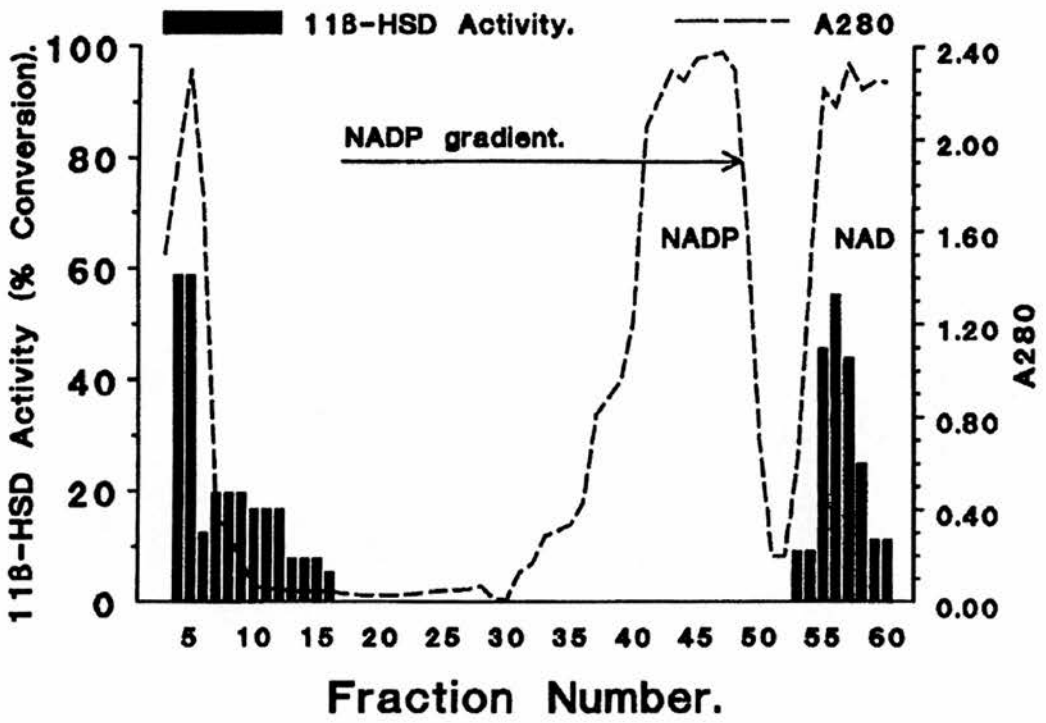


Fig.4.5 Gradient elution off AMP matrix with cofactor. CHAPS solubilised human placental fractions were loaded onto the AMP affinity column and the relative efficiency of elution by NADP, NAD and 5'AMP was determined gradient elution. Findings with NADP gradient illustrated. Fractions: 1-5, flow through/wash; 6-15, wash; 16-48, increasing NADP gradient 0-3000μM; 48-50, wash; 51-60, 3mM NAD elution. In the equivalent scheme for NAD and AMP gradients 11ß-HSD eluted beginning at fraction 29 (30μM) and 38 (300μM) respectively (not shown).

HSD(dehydrogenase activity), which is in contrast with the abundant rat liver 11ß-HSD(reductase activity)[28,40]. Secondly, the subcellular localisation of the 11ß-HSD appears different. Rat liver 11ß-HSD is localised to microsomal and 'nuclear' fractions[28]. By contrast, our preliminary work suggests a different and more widespread subcellular localisation of placental 11ß-HSD (see Chapter 3) with activity abundant in the microsomal (110000g pellet) and mitochondrial/ heavy microsomal (25000g pellet) fractions. Further differential centrifugation of the human placental 25000g fraction produced "mitochondria enriched" and "microsome enriched" sub-fractions with similar 11ß-HSD activity. Thirdly, rat liver microsomes were found to have substantial 11ß-HSD activity in absence of added cofactor, being increased less than two-fold by 400µM NADP. In contrast, placental 25 000g membrane pellets had little 11ß-HSD activity in the absence of added cofactor, but this was increased ten-fold by 400µM NAD. The cause of this difference is unclear, though the higher pyridine nucleotide cofactor concentrations in liver[170] and the relatively low K_m of liver 11ß-HSD for NADP (0.196µM) [35] would cause any NADP sequestered in the microsomes to have a potent influence. The apparent K_m of human placental 11ß-HSD for NAD is considerably higher (13µM) making the enzyme less sensitive to trace amounts of endogenous cofactors and causing it to show a clearer cofactor preference. In intact cells NAD is more abundant than NADP[170] hence both enzymes are suited to respond to physiological cofactor levels. Fourthly, our preliminary data suggest that their immuno-reactivity to anti-'liver-type' anti-sera is very different. In conditions that cause minor immuno-neutralisation and major immuno- precipitation of solubilised liver microsomal 11ß-HSD, the solubilised placental 11ß-HSD showed absolutely no immuno-neutralisation or immuno-precipitation of the 11ß-HSD activity with either 56-125 or 56-126 anti-sera[38](gift of Dr Carl Monder, Population Council, New York).

4.3 Discussion.

The existence of a second enzyme (11ß-HSD2) has been the subject of much speculation[167], largely based on evidence of the presence of 11ß-HSD activity in the absence of liver type 11ß-HSD protein or mRNA. In addition, variant mRNAs which hybridise to rat liver 11ß-HSD cDNA have been identified in kidney[39] and colon[46,54].

Further experimentation, however, has shown these renal mRNA variants to be derived from differential promoter usage of the characterised liver 11ß-HSD gene[46] and may encode proteins with no demonstrable 11ß-HSD activity[57,61]. This has implications for *in situ* hybridisation results on renal cortex[13,44] as hybridisation to 'liver type' 11ß-HSD probes in kidney may be detecting variant mRNA species without the potential for translation to active 11ß-HSD enzyme. Antibodies to 'liver-type' 11ß-HSD also detect what appear to be additional tissue-specific proteins (e.g. 26K in brain, 40K in kidney and 47K in testis) with strong antigenic similarities to 'liver-type' 11ß-HSD[38]. Indeed, when attempts have been made to separate such proteins (26K species) from the 34K liver-type 11ß-HSD protein the variant protein had no demonstrable activity[45]. Thus the evidence suggests 11ß-HSD2 is the product of a separate gene and is not, therefore, readily detectable by antibodies or nucleic acid probes specific for 'liver-type' 11ß-HSD. From the evidence presented here it seems clear the placental isoform represents an 11ß-HSD enzyme distinct from the 'liver-type' 11ß-HSD. It seemed more than coincidence that both placental 11ß-HSD and the isoform that had been reported in renal distal nephron (rabbit cortical collecting duct):- (i) are NAD-dependent; (ii) have a K_m for physiological glucocorticoids two orders of magnitude lower than liver 11ß-HSD (14) and (iii) appear not to be immuno-reactive to 'liver-type' anti-sera[15]. The fact that kidney 11ß-HSD activity bound specifically to the same 5'AMP affinity column as placental 11ß-HSD strongly supported the conclusion that these tissues contained very similar or identical 11ß-HSD isoform(s) [180].

In summary we find strong evidence for an NAD-dependent 11ß-HSD isoform in both human and rat placental tissue which is distinct from the previously characterised enzyme in rat liver and may be encoded by a separate gene. Henceforth, in this thesis, this isoform in placenta is referred to as 11ß-HSD type 2 or 11ß-HSD2.

Chapter 5 : Development of a Novel Method Allowing Highly Specific Affinity Labelling of 11ß-HSD2.

5.1 Introduction.

The kinetic studies of 11ß-HSD2 purified by AMP-agarose chromatography from human placenta revealed a very low K_m for corticosterone (14 1 nM) (see Chapter 4). This is an uncommonly low figure for the K_m of an enzyme for a major substrate and is getting down to the range of the K_ds of receptors for their ligands. As purification of placental 11ß-HSD2 was revealing that when the enzyme was purified significantly beyond 1000-fold, its activity was labile and prone to inactivation on further purification procedures (see Chapter 6), it was clear that it would be a major advantage to be able to track the enzyme even if inactive. Receptors can often be affinity labelled by ligands (or their homologues) which bind them with high affinity; having a K_d in the low nM range. Thus, because of the receptor-like K_m of human placental 11ß-HSD2 for corticosterone, affinity labelling with this steroid was successfully attempted using the following method.

5.2 Results.

5.2.1 Method developed for UV Photoaffinity Labelling and its analysis by Fluoroautoradiography.

The standard method developed used a reaction volume of 600µl irradiated by intense UV light in the wells of a 24 well plate (diameter 1.5 cm) with the lid off. 467µl of tissue sample diluted in buffer C was added to wells of 24 well plates (diameter 1.5cm) and maintained at 37°C for 3 min. Addition of 60µl of 250mM dithiothreitol, 48µl of 5mM NAD (both in buffer C) and finally ^{3}H-steroid (in 25µl of 10% ethanol : buffer C) completed the reaction giving a final concentration of 50nM ^{3}H-steroid in 600µl, unless otherwise stated. In the experiments presented in this Chapter (to minimise UV damage to protein), reactions were placed in UV light (312nm transilluminator at distance of 5cm above plate; lid off) for 15 min at 37°C. The procedure was easily scaled up. Similar labelling occurs at 254nm and is even stronger at 0°C or with longer UV exposure. However, all three of these variations also induce formation of

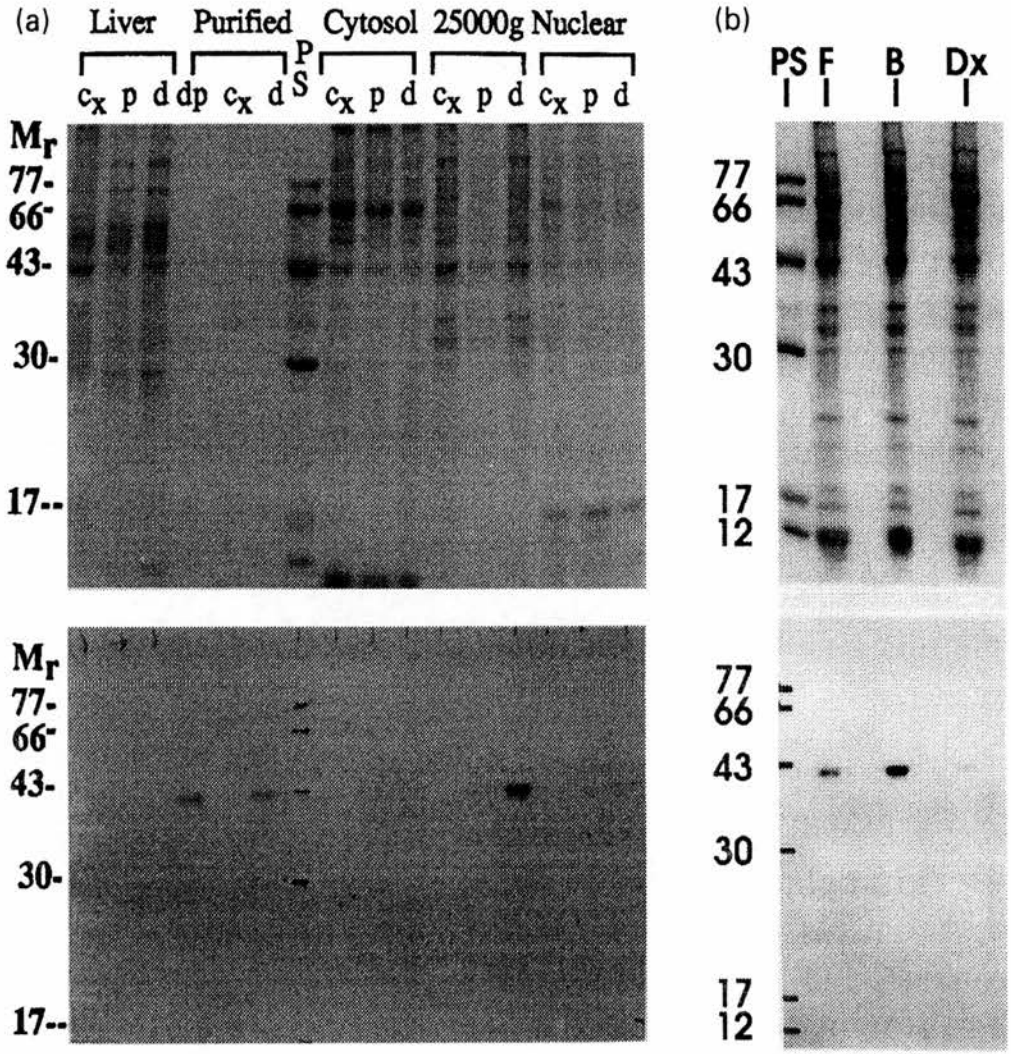


Fig.5.1 Native photoaffinity labelling of 11ß-HSD2. The bottom panels are autoradiographs of labelled protein samples run on the 12.5% SDS-PAGE gels shown in the top panels (stained with Coomassie blue). Apparent M_r (x1000) of protein standards (**PS**) indicated to *left side* of gels. **(A)** Triplicate samples of rat liver microsomes, AMP chromatography purified human placental 11ß-HSD (similar to fraction 6 in fig 3.4(b)) and three human placental subcellular fractions. All samples were photoaffinity labelled simultaneously with ^{3}H corticosterone in the presence of 400µM NAD (**d** at *top of lane*), 400µm NADP (**p**), both cofactors at 400µM (**dp**) or both cofactors and 50µM carbenoxolone (**cx**). A single protein band at 40k is labelled in an NAD-dependent, carbenoxolone-blockable fashion. **(B)** Samples of human placental 25000g pellet (mitochondria + heavy microsomes) photoaffinity labelled simultaneously in the presence of 400µM NAD and ^{3}H-cortisol (**F**), ^{3}H-corticosterone (**B**) or ^{3}H-dexamethasone (**Dx**). Protein loading <0.5µg for "purified" lanes , otherwise 30µg/lane in studies **(A)** and **(B)**.

minor bands below 40k and protein damage with prolonged 254nm UV exposure will be more extensive.

During analytical work leading to identification of the 11ß-HSD2 protein, duplicate labelled samples were acetone precipitated and resolved by SDS-PAGE[83], or 2-D electrophoresis[87,88] (see Methods: Chapter 2). One of the finished gels was stained with Coomassie Blue to allow detection of major proteins and molecular weight standards and was processed for fluoroautoradiography in 'Entensify' solutions and vacuum dried before exposure to film. The other was silver stained allowing identification of all the proteins (major and minor) to assess the extent to which the labelled 11ß-HSD2 protein had been fully resolved from contaminants (see Chapter 6)

5.2.2 Characteristics of the photoaffinity labelled protein identify it as 11ß-HSD2.

As fig.5.1 shows, a protein with all the expected characteristics of 11ß-HSD was uniquely photoaffinity labelled. This protein was 40k in size, present in the subcellular fractions with 11ß-HSD activity (25000g pellet> 750g pellet (nuclear/debris) 110000g pellet (light microsomes, not shown)), but absent in cytosol. The labelling was NAD-dependent, independent of NADP and blocked by carbenoxolone. Labelling of fractions after AMP-agarose chromatography showed the labelled protein exactly followed 11ß-HSD activity, being present when 11ß-HSD eluted with NAD (e.g. fig.6.1(b). fractions 87-120), and in the flow-through (fractions 1-30), but absent in the inactive wash fractions. Moreover, the same protein could be affinity labelled with cortisol and dexamethasone (corticosterone>>cortisol>>dexamethasone: fig.5.1(b)), but there was no labelling with aldosterone. This novel technique appears to result in completely selective labelling of placental 11ß-HSD by glucocorticoids, with a potency which parallels their affinities as substrates for the enzyme. Using this technique it was possible to track the 11ß-HSD2 protein after activity had been lost, so allowing final purification by 2-D electrophoresis.

5.3 Discussion.

The affinity labelling technique developed here is unusually straightforward and specific for 11ß-HSD2. Photoaffinity labelling is more commonly described for *receptors and binding proteins* and a few such procedures report labelling by unmodified steroid compounds acting

as ligand (e.g. GR by dexamethasone) but even in such cases these 'common' ligands lack the desired specificity and are replaced by compounds developed to allow greater specificity (e.g. RU26988 and RU28362 for exchange assays/photoaffinity labelling specific for GR[9,100]). Affinity labelling of *enzymes* is much less commonly by photoactivation and more often achieved using a specifically designed activatable compound containing a reactive functional group (usually electrophilic e.g. bromoacetate) which is attacked by an activating substituent (usually a nucleophilic side chain of an amino acid) in the enzyme's active site (e.g. 3-methoxyestriol 16(bromo[2-^{14}C] acetate) labelling homogeneous placental 17ß-HSD type 1 at two histidines in its active site[96]). Less commonly, enzymes can be *photo*affinity labelled by precisely chosen synthetic compounds which, when photoactivated, develop short-lived highly reactive substituents which label the target enzyme if a suitable amino acid residue is nearby (e.g. UV induced labelling by 19 nortestosterone acetate of purified ^{5}-3 ketosteroid isomerase from *Pseudomonas testosteroni*[177]). What makes the labelling technique described here particularly unusual is that it can use a physiological substrate, in a crude tissue extract, containing thousands of different proteins (in contrast to the two examples, given above, using pure enzyme), and label the enzyme for that substrate with great specificity (analysis in Chapter 6 reveals only a single labelled spot on 2-D electrophoresis). To our knowledge, the only other study describing photoaffinity labelling of a steroid metabolising enzyme by what may be a natural substrate is the labelling of Pseudomonal ^{5}-3 ketosteroid isomerase, with testosterone. This, however is a less avid label than synthetic 19-nortestosterone acetate. Though the parallels are intriguing, that study[176,177] utilised pure enzyme and 500-fold higher steroid concentrations than employed here for 11ß-HSD2 labelling.

Several findings suggest the affinity labelling procedure, described above, occurs within the steroid binding pocket of the active site. Firstly, the affinity labelling is NAD-dependent as is 11ß-HSD2 activity. There are a few reports of cofactor-dependent affinity labelling of other dehydrogenases. NAD-dependent lactate dehydrogenase is affinity labelled, in the presence of NAD, by bromopyruvate on the major catalytic residue (histidine 195)[175,179]. Secondly, carbenoxolone, an 11ß-HSD2 inhibitor, blocks labelling, as does a 1000-fold excess of cold corticosterone. Thirdly, the rank order of potency for glucocorticoid labelling correlates with the substrate affinities of human placental 11ß-HSD2.

Finally, the glucocorticoid groups most likely to participate in covalent photoaffinity labelling of 11ß-HSD2 are the carbonyl group at C3 and the C11 position itself. Labelling via the C20

carbonyl appears less likely as it is expected to be less photoexcitable and as covalent attachment of the C20-C21 grouping (following the common C17-C20 cleavage) would not attach any radioactivity, as the steroids are tritiated at C1,2,6 and 7 only. The C3 carbonyl would be highly photoactivated to produce both alkoxy radicles and reactive ketenes, both would react relatively indiscriminately[2], potentially with almost any closely approximated amino acid, but especially with reactive cysteine, lysine, histidine or tyrosine residues. The photoreactive state of C11 is harder to anticipate but likely to be greatly heightened in the active site where it will make the transition to a C11 carbonyl, a photoexcitable group in its own right.

Chapter 6 : Purification to Homogeneity and Determination of Amino Acid Sequence of 11ß-HSD2.

6.1 Introduction.

Having identified a means of solubilising human placental 11ß-HSD2 and an affinity chromatography matrix allowing 1000-fold purification, the aim was now to obtain amino acid sequence from the 11ß-HSD2 protein. Thus further purification was required to allow isolation of homogeneous protein.

6.2 Results.

6.2.1 Refining the AMP Affinity Chromatography Procedure.

Firstly, the N6 5'AMP affinity chromatography procedure was optimised. Throughout this optimisation the 40kDa band originally observed (Chapter 3) continued to be the only one consistently segregating with 11ß-HSD2 activity. It was found that a purification of several thousand-fold could be achieved in this one step using a small (1ml) column of this 5'AMP-agarose and resulted in small volumes of eluate where the 40k protein was the only significant protein detected. However, by this degree of purification the 11ß-HSD2 activity was very labile and easily inactivated. Chromatography for highest purity (e.g. lanes A1 + A2, fig.6.1(a)), used the 'analytical' method detailed in Chapter 2 (Methods: Affinity chromatography) but suffered from reduced reproducibility and low yield of active 11ß-HSD. It was clear these losses were unacceptably high when the procedure was tried at preparative scale using a 30ml AMP-agarose column. Accordingly a second set of optimisations were carried out for preparative scale purification. This revealed higher yield and higher purification was possible with 6 x 5ml columns run in parallel rather than a single 30ml column. The optimised 'preparative' procedure is as outlined in Chapter 2. This preparative scale method would reliably yield 80-100ml of eluate having high 11ß-HSD2 activity (50µl giving >90% conversion in 10 mins of 12nM ^{3}H-corticosterone during a standard 11ß-HSD2 assay).

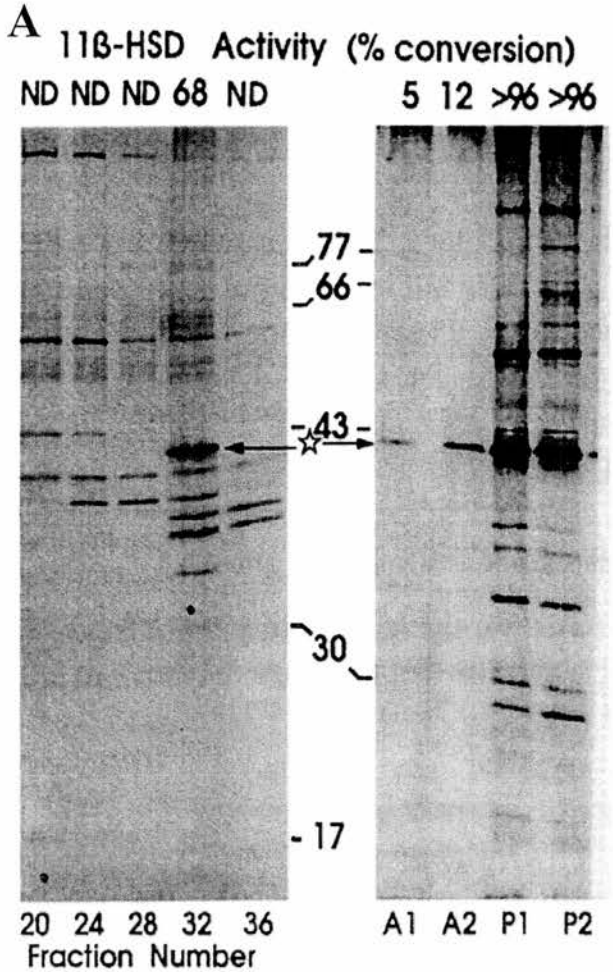
6.2.2 Purification Beyond AMP Affinity Chromatography.

These large scale preparative conditions (see Chapter 2 and fig.6.1(b)) reproducibly gave 20-fold higher yield (≈35%) at somewhat reduced, though still 1000-fold, purification (fig6.1(a) fractions P1 and P2). Note the 40k band (starred) is the most prominent but contamination persists at a range of molecular weights, notably several narrow bands very close to (especially above) 40k. These closely flanking bands indicated contaminating proteins that were likely to defeat further purification steps based solely on resolution by size (e.g. gel filtration).

Accordingly separation by charge was attempted using ion exchange chromatography. Conditions using DEAE-Sepharose were determined allowing >90% yield and 5-8 fold purification of CHAPS-solubilised placental 25000g pellet 11ß-HSD2 activity. However, when this was tried, either before or after the preparative affinity chromatography the yield of active enzyme fell to <2%. This loss of activity was intractable to alteration of conditions and it was clear the solubilised, highly purified placental 11ß-HSD2 enzyme eluting off AMP affinity chromatography was too labile to withstand significant further purification procedures. At this stage efforts were made to find a method of tracking the enzyme when inactive and the highly successful affinity labelling procedure was rapidly developed (see Chapter 5). This allowed the enzyme to be tracked after activity had been lost in a fashion which withstood harsh denaturing conditions. Thus it was decided to further purify the placental 11ß-HSD2 by separating by both size and charge using 2D electrophoresis having affinity labelled a portion of the enzyme between AMP affinity chromatography and 2D-electrophoresis.

6.2.3 2-D Electrophoresis.

Fractions containing enzyme eluted from AMP-agarose were affinity labelled with ^{3}H-corticosterone and then subjected to conventional 2-D electrophoresis (IEF + SDS-PAGE). After prolonged exposure gel autoradiography remained negative. Accordingly, NEPHGE gels were run. A strong signal, indicating the presence of a single affinity labelled protein spot of basic pI (pI 9.1) and apparent molecular weight of 40k, was seen on autoradiography (fig.6.2(a+b)). It was notable that this was accompanied by other proteins close to 40k which were not affinity labelled. NEPHGE conditions were optimised (fig.6.2) to allow preparative scale gels with substantially increased yield and reduced smearing of the affinity labelled protein, which was otherwise a major problem. The optimised procedure allowed preparation



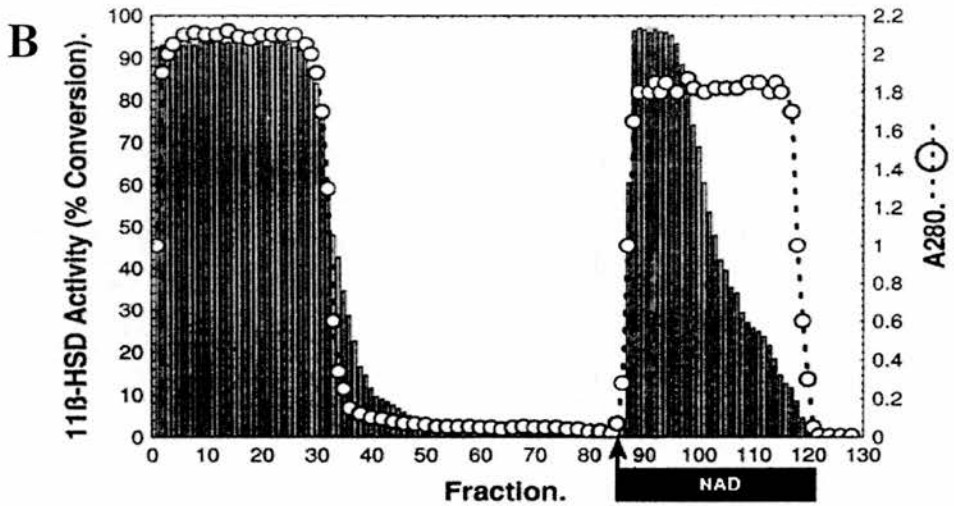


Fig.6.1 Optimised 5' AMP affinity chromatography for purification of 11ß-HSD2. **(A)** 12.5% SDS-PAGE gels (silver stained) of fractions from 5'AMP chromatography with 11ß-HSD2 activity (percentage conversion of 12nM ^{3}H-corticosterone in 10 mins) indicated *above lanes*, position of 11ß-HSD2 protein (starred arrow) and M_r. (x 1000) shown *between* panels. Left panel, preliminary analytical study showing samples before, during and after harsh elution with NAD (fractions 31-34). Right panel, samples from two particularly clean analytical runs (A1 + A2) and pooled high activity fractions from two typical preparative runs (P1 + P2), as described in Materials and Methods. For analytical runs 190µl of fractions used in standard 11ß-HSD assays, 1 ml (acetone precipitated) for SDS-PAGE: in preparative runs 50µl (assays) and 250µl (SDS-PAGE) were used. **(B)** Chromatographic profile of preparative scale run showing 11ß-HSD2 activity (bars) in relation to A_{280} (protein concentration or NAD on elution) of fractions (5 ml) of common outflow of six 5 ml AMP-agarose columns run in parallel. Fractions 1-36 loading, 37-86 washes, 87-120 elution with 1 mM NAD (the most active of these being equivalent to samples P1 and P2 shown in **(A)**.

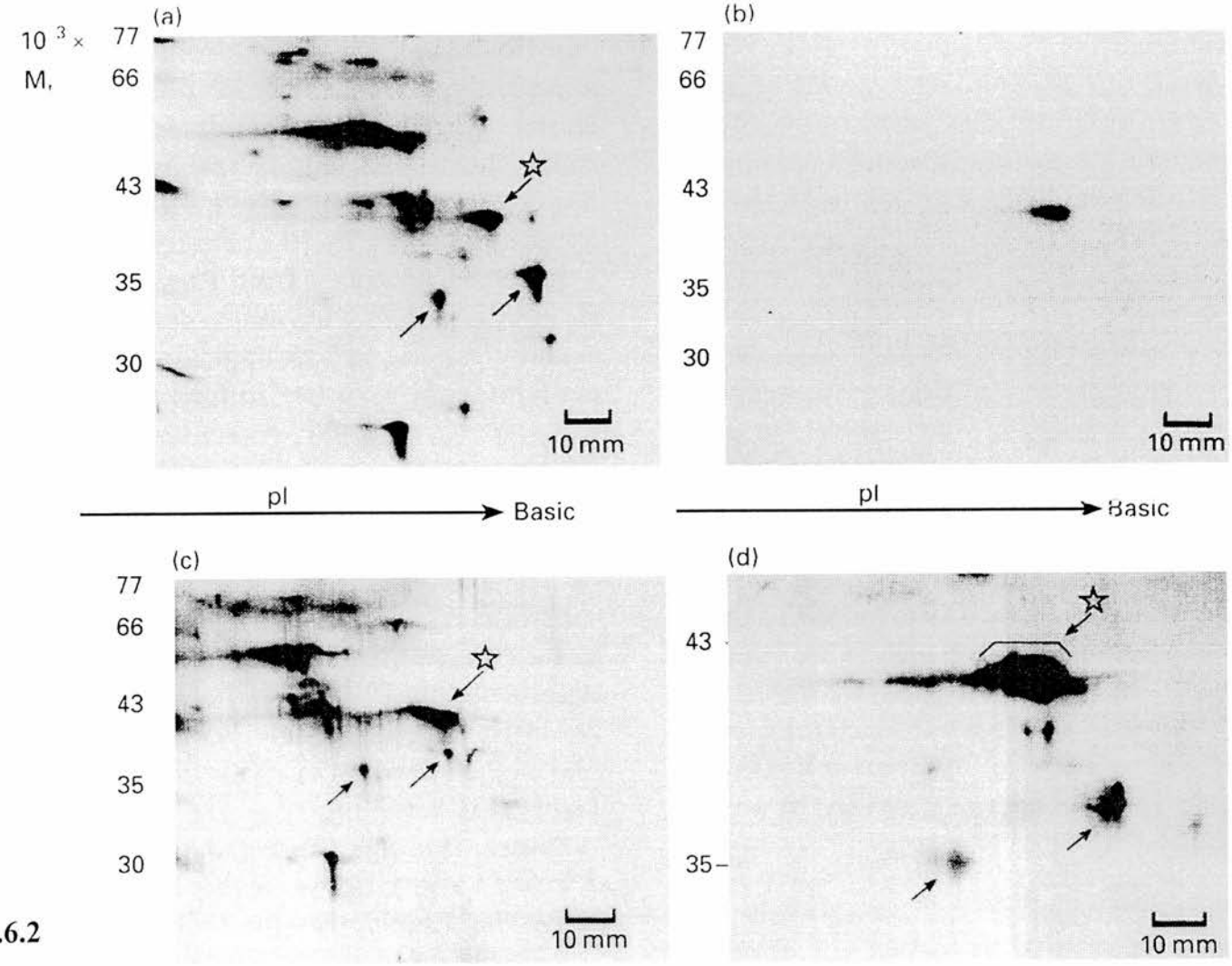


Fig.6.2

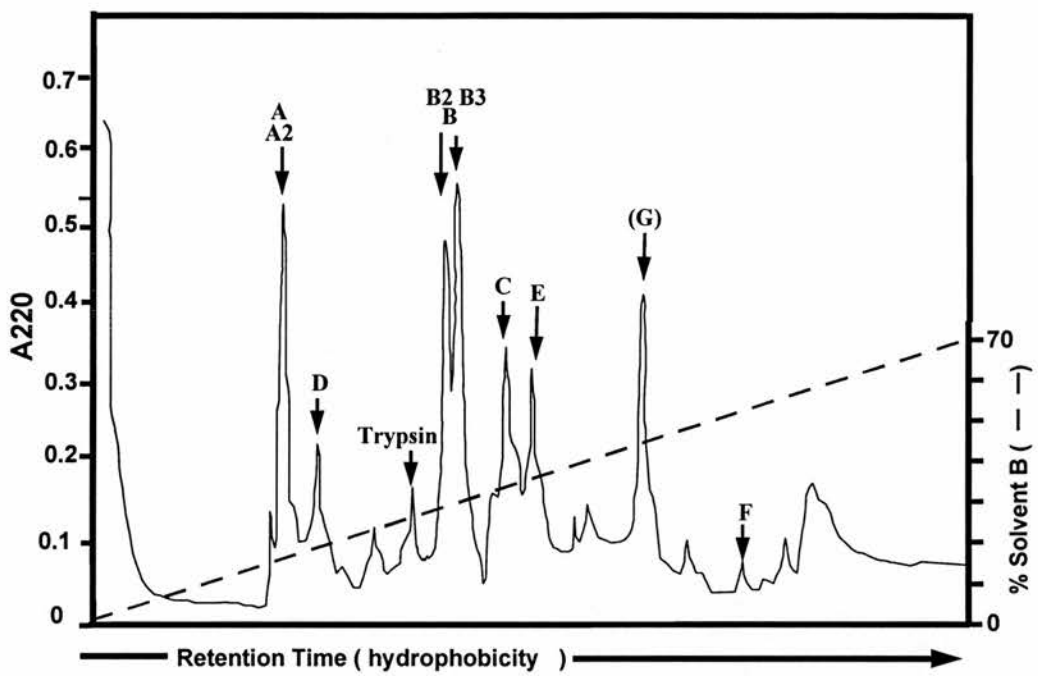


Fig.6.3 Microbore HPLC profile of 11ß-HSD2 tryptic digest. *Solid line* indicates elution of peptide peaks (detected by A_{220}, highest peaks corresponding to 500pmol). The elution position of trypsin and peaks from which peptide sequence was obtained (G by V8 sub-digest) are indicated. The digest was loaded onto the HPLC RP300 column equilibrated in solvent A (0.1% TFA, water) and developed with a linear gradient (*dashed line*) of 0-100% solvent B (0.08% TFA, 80% acetonitrile/water) over 45 min at 200µl/min.

CYCLE	SEQUENCED PEPTIDES [Residues, Yield(pmol)].																			
	A		A2		B			B2		B3		C		D		E		F		G	
1	N	445	L	190	A	180	412	L	315	V	126	I	55	V	411	?	?	?	?	V	45
2	V	426	P	163	L	96	405	L	330	S	119	V	36	L	364	L	406	L	16	N	29
3	G	383	V	182	Q	83	354	Q	318	I	117	T	16	E	231	L	307	D	9	F	27
4	Q	311	A	180	P	76	339	M	316	I	117	V	19	F	333	Q	238	S	7.5	F	15
5	W	>60	T	112	G	66	281	D	170	Q	85	G	15	T	74	L	214	ML	?	G	10
6	E	195	R	31	Q	57	271	L	243	P	46	SI	?	K	37	L	221	G	5	A	9
7	K	97			P	71	243	T	134	G	40	P	15			R	21	F	6	L	7.5
8					G	62	197	K	135	C	40	A	16					TD	?	E	2.5
9					T	39	135	P	170	F	41	G	14					V	8		
10					T	40	116	G	175	K	8	D	12					L	5.3		
11					P	40	121	D	120			M	13					A	3.8		
12					P	33	92	I	167			P	11					T	3		
13					Q	30	82	S	60			Y	10					VP	?		
14					D	18	43	R	28			P	10								
15					A	23	62					-	0								
16					A	25	71					L	8.4								
17					Q	15	43					G	7.4								
18					D	11	16					A	8.1								
19					P	10	21					Y	3.5								
20					N	10	17					G	2.5								
21					L		13					T	1.8								
22					S		6					S	2								
23					P		9					K	1.5								
24					G		9														
25					P		7														
26					S		3														
27					P		3														

Table.6.1 11ß-HSD2 Peptide Sequence. The amino acid and yield (in pmol) obtained on sequencing 11ß-HSD2 peptides A-G are given by cycle number. Peptide B was sequenced twice (using a second sample also from tryptic digestion of purified 11ß-HSD2). Cycle C(15) produced no residue signal. Cycles F(5), F(8), F(13) and C(6) produced two residues at similar yield making assignment difficult. Cycle A(5) is definitely W but the yield is imprecise. Note *boxed sequence* in the C peptide is a classical short chain alcohol dehydrogenase motif indicating the likely active site.

of over 40μg of homogeneous 11ß-HSD2 protein which was blotted onto PVDF (Problott) membranes.

6.2.4 Amino Acid Sequence.

PVDF blots were stained with amido black and the 40k target protein accurately excised. Sequencing of PVDF pieces revealed homogeneous 11ß-HSD2 was N-terminally blocked. This is unlikely to be an artifact of the purification as, on sequencing, an amino acid signal was completely absent (rather than just low), and we have obtained high yield N-terminal sequence from other proteins blotted from gels in the same fashion. *In situ* tryptic digestion of approximately 30μg of 11ß-HSD2 protein produced multiple peptides and several were sequenced, yielding in total, over 100 residues of amino acid sequence (fig.6.3 + Table 6.1). Peptide G was blocked. This was sub-digested with *Staph. Aureus* V8 protease yielding a sequencable daughter peptide.

6.2.5 The Overall Purification Procedure.

Table 6.2 summarise the steps in the purification procedure we have described that allowed isolation of homogeneous 11ß-HSD2 protein from human placenta (>16000 fold purification), and direct determination of over 100 residues of its internal amino acid sequence. In Chapter 7 it is described how the amino acid sequence derived was used to clone a full length 11ß-HSD2 cDNA from a human placental library which, on transfection into mammalian cells, expresses 11ß-HSD2 activity and a 40k protein which can be affinity labelled (see Chapter 8) by the method described above in Chapter 5.

6.3.Discussion.

Several findings suggest the 11ß-HSD2 membrane environment has polar aspects. Thus, moderate ionic strength stimulates activity (Table 3.1), the most hydrophobic lipid-like detergents (e.g. Tween 20) are not the most useful for preserving 11ß-HSD2 activity and the 11ß-HSD2 protein has a very high pI (pI>9). The very basic pI (pI =9.1) is surprising. Proteins this basic are unusual, often being ribosomal or associated with nucleic acid (chromatin or RNA). Rarely are they intrinsic membrane proteins and this combination suggests interactions of 11ß-HSD2 beyond NAD and steroids, perhaps with other membrane proteins or charged lipid. Thus it may well be that interactions of this nature (especially phospholipid head groups)

	HOMOGENATE	SUBCELLULAR FRACTIONATION	SOLUBILISATION	AMP-AGAROSE	2D GEL.	BLOT	ELUTED PEPTIDES	INITIAL SEQUENCING YIELD
PROTEIN (10 PLACENTAS)	46.8g	5.22g	1.85g	640µg	43µg	30µg	≈520pmol (≈21µg)	Best yield = 445pmol
STEP YIELD	——	67%	13%	36%	47%	70%	Best = 70%	85%
CUMULATIVE YIELD	100%	67%	8.7%	3.15%	1.47%	(1.05%)	(0.75%)	——
STEP PURIFICATION	——	6	0.367	1040	>7	——	——	——
CUMULATIVE PURIFICATION	——	6	2.2	2290	>16000	——	——	——
ACTIVITY, nmol/(min.mg protein)	0.025	0.15	0.055	57.3	——	——	——	——
RADIOACTIVE TRACER	——	——	——	——	100%	70.2%	49.8%	——

Table.6.2 Summary Of Purification Of 11ß-HSD2. Purification using 10 placentas yielding 43µg of 16000-fold purified 11ß-HSD2. Affinity labelling of tracer amounts of 11ß-HSD2 after AMP-agarose chromatography allowed a means of assessing yields beyond this stage. 11ß-HSD2 peptide recovery (following *in situ* tryptic digest, elution off PVDF and microbore HPLC) was generally lower with increasing peptide hydrophobicity (see fig.6.3).

are involved in stabilising the enzyme, as occurs with the related NAD-dependent, short-chain alcohol dehydrogenase (SCAD) enzyme, 3-hydroxybutyrate dehydrogenase[95]. We have not investigated this further as development of the affinity labelling technique allowed purification of 11ß-HSD2 to homogeneity, when activity was not maintained.

All peptide sequence obtained was unique, confirming 11ß-HSD2 as a novel protein. This is the first directly determined 11ß-HSD2 peptide sequence from any source. Direct amino acid sequence allows information about amino acid order and post-translational modification and avoids some pitfalls of cDNA-predicted amino acid sequence, such as use of rare codons (e.g. selenocysteine). There is one potential N-linked glycosylation site (NLS: peptide B) which in the native protein appears to be largely free of glycosylation, at least in human placenta, as the amino acid sequence yield did not drop across the N residue (asparagine). Peptide C contains a classical short chain alcohol dehydrogenase (SCAD) motif (boxed in Table 6.1, peptide C) indicating 11ß-HSD2 belongs to this enzyme superfamily. There was one clear 'blank' cycle in the middle of peptide C (cycle 15); it is possible a modified amino acid naturally occurs here. Further analysis of the 11ß-HSD2 sequence is presented in Chapter 7.

Chapter 7 : Cloning of Human Placental 11ß-HSD2 cDNA and Analysis of the Predicted Structure of the Encoded Enzyme.

7.1 Introduction.

Having obtained extensive amino acid sequence from homogeneous 11ß-HSD2, two objectives then fell within reach:- firstly to raise a specific anti-11ß-HSD2 antibody, which is dealt with in Chapter 9 and secondly to clone the cDNA encoding 11ß-HSD2 in placenta so also allowing prediction of the entire amino acid sequence of the enzyme, which is dealt with in this Chapter.

7.2 Results and Discussion.

7.2.1 Cloning of 11ß-HSD2 from Human Placenta.

As amino acid sequence had been obtained from several distinct peptides it was decided to follow a PCR strategy whereby primers corresponding to the peptide sequences would be used in pairs to amplify the fragments of the 11ß-HSD2 cDNA spanning the peptide pair. Extensive searches of the protein databases were made with the peptide sequences but were unhelpful in giving definite information about the NH2 to COOH order they occupied in the 11ß-HSD2 protein. Thus the five peptides about which there was most certainty in the amino acid sequence (peptides A, B, C, D and B2) were chosen and the region giving the least degenerate primers were selected and the corresponding degenerate primers for both top and bottom DNA strands were synthesised. Where there was a four-fold degeneracy an inosine was put in the primer sequence. Placental RNA was prepared, extensively DNAse treated and reverse transcribed to make the placental cDNA on which the degenerate PCR amplification was carried out.

The degenerate primers were used in all top-bottom pairwise combinations in an initial PCR screen carried out on human placental cDNA Combinations involving primer Bb resulted in the amplification of several particularly prominent DNA products, the majority of which appeared to be independent of the top primer used (fig.7.1 lanes1-4), and indeed PCR with Bb alone reproduced many of these bands. A clear exception was the PCR product arrowed in fig.7.1 (lane 2), this was specific to the CtBb primer pair and became the major product (fig.7.1 lane 5) using more stringent PCR conditions. This CtBb fragment was directly cloned and sequenced revealing a single open reading frame spanning its length (531bp) and

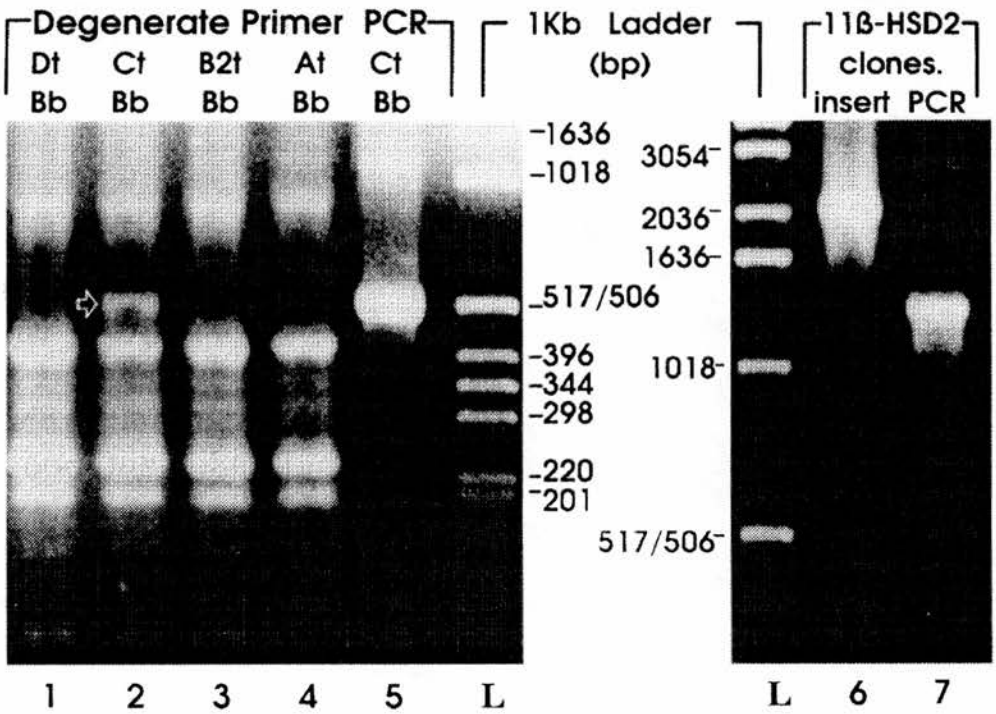


Fig.7.1 PCR to amplify 11ß-HSD2 sequences. Ethidium bromide stained agarose gel showing analysis of PCR amplifications. **Lanes 1-5** RT-PCR on human placental mRNA using degenerate primers based on 11ß-HSD2 peptide sequences A-D. Primers used (Xt = top, Xb = bottom, X = peptide) indicated *above corresponding lane*. Lanes 1-4 initial PCR conditions used, lane 5 using more stringent amplification yielding only the CtBb specific product (531bp) identified initially (*arrowed*, lane 2). **Lanes 6 + 7** PCR (using vector- specific primers flanking the cloning sites) of 11ß-HSD2 inserts of 1179bp partial pcDNA1 library clone (1157bp + 22bp polyA: lane7) and the 1919bp (1897 + 22bp polyA) pDR2 clone containing the full coding region (lane 6). **Lane L** = 1kb ladder (Gibco-BRL.)

with a predicted amino acid sequence encompassing 4 11ß-HSD2 peptides (C, B, A and B3) including a short chain alcohol dehydrogenase (SCAD) motif (YXXXK, see below). This CtBb fragment was used (a) to design a synthetic peptide to raise an anti-11ß-HSD2 antibody (see Chapter 9) and (b) to isolate a complete 11ß-HSD2 cDNA.

A human placental cDNA library in pcDNA1 was initially screened by a PCR based approach and a 1177bp partial clone isolated (lane7, fig.7.1) which contained the CtBb fragment, 45bp further 5' and 603bp further 3' (reaching to the 3'end of the cDNA with a 22bp polyA tail). Conventional screening of 700 000 plaques from a human placental DR2 library (Clontech), using the incomplete 11ß-HSD2 sequence as a probe, allowed isolation of a single clone which, when converted to pDR2 plasmid form (see Methods, Chapter 2), produced very abundant, NAD-dependent, 11ß-HSD2 activity in transfected CHO cells. This clone was designated p11ß2.

7.2.2 Characteristics of the cDNA clone and Predicted 11ß-HSD2 Protein Sequence.

7.2.2.a General features of the 11ß-HSD2 cDNA clone.

This active 11ß-HSD2 clone, p11ß2, contained a 1897bp cDNA with 22bp polyA tail (1919 bp total), and has a G+C rich composition (63.25%). The 5' end of the cDNA (first 385 bases) is extremely G+C-rich (79.2%) and contains a 'CpG island'[111] with 56 occurrences (in 385 bases) of this usually scarce dinucleotide. G+C and CpG content fall abruptly beyond the region (to 55% G+C, while CpG frequency falls 4-fold). These features, which extend to the start of the 11ß-HSD2 cDNA, are suggestive of a 5' transcription regulatory region. The best candidate for initiation of translation is the ATG codon beginning 134 bases from the 5' end of the cDNA (set to +1 in fig.7.2), this is in a good context for initiation of translation, with 9/10 matches to the putative ideal ribosome binding site (GCCGCCATGG)[174], and is within the longest open reading frame (-63 +1215:fig.7.2) defining a predicted coding region of 1215bp (+1 1215), flanked by a 5' untranslated region (5'UTR) of 133bp (-133 -1) and long 3'UTR of 549bp (1216 1764). The predicted coding region encodes a 405 amino acid protein (calculated mol. wt. 44126) encompassing all the 11ß-HSD2 peptide sequence derived from digests of the purified protein (boxed in fig.7.2). The most abundant amino acids are leucine (68 in 11ß-HSD2), alanine(46), glycine(32), which are all highly represented in proteins in general, followed by proline(32) and arginine(30). The high arginine content seems responsible for the very basic nature of the 11ß-HSD2 protein, so clear during purification, as basic residues (R=30, K=12, H=7) markedly outnumber acidic ones (D=15, E=15), giving a

Fig.7.2 cDNA and predicted amino acid sequence of human placental 11ß-HSD2. **(A)**cDNA numbering, -133 1764 (*to side of DNA sequence*) sets +1 at start of predicted coding region (note the 'no base zero', convention is followed). The ATG initiation codon and polyadenylation motif are double underlined. *Dashed line* encloses the 531bp CtBb fragment identified by RT-PCR (fig.1 lane 5). Boxed sections of predicted amino acid sequence are those of the 10 peptides sequenced from tryptic digests of purified human placental 11ß-HSD2; peptide nomenclature (small boxes) and numbering of amino acid sequence are *to side of sequence (amino acid sequence numbers are underlined)*. Circled residues are Cys227 (unidentifiable residue on amino acid sequencing of C peptide) and Asn394 (the only potential N-glycosylation site in 11ß-HSD2, likely unused in placenta). **(B)** Predicted secondary structure of human placental 11ß-HSD2 and 3α20ß-HSD from *Streptomyces. hydrogenans.* Segments represented as white (-helix, up from line or ß-sheet, down from line) and black (loops, close to line) are of highest certainty, grey are of moderate certainty whilst segments represented by a flat line cannot be predicted with moderate certainty. (**C**) Alignments of 12 enzymes with sequence and functional similarity to human 11ß-HSD2. On *left* percentage amino acid identity with regions (I-IV) of human 11ß-HSD2 is indicated by larger font figures (small figures indicate amino acid residue numbers at start of regions). On *right* alignment of 3 motifs highly conserved in SCADs, with $(X)_n$ indicating n amino acids separate the first residues of the adjacent motifs. Enzymes are: h- and s11ß2, human and sheep 11ß-HSD type2; h17ß1/2/3, human 17ß-HSD types 1, 2 and 3; p17ß4, pig 17ß-HSD type 4; b11retdh, bovine 11-cis retinol dehydrogenase, h3hbdh, human 3-hydroxybutyrate dehydrogenase, h11ß1, human 11ß-HSD type 1; C.s7αhsd, 7 -HSD from *Clostridium. sordellii.*; 3α20ßhsd, 3α20ß-HSD from *Streptomyces. hydrogenans.*; pCarbred, pig carbonyl reductase; h3ß2, human 3ß-HSD type 2 (the adrenal/ovarian isoform).

(See Figure Overleaf).

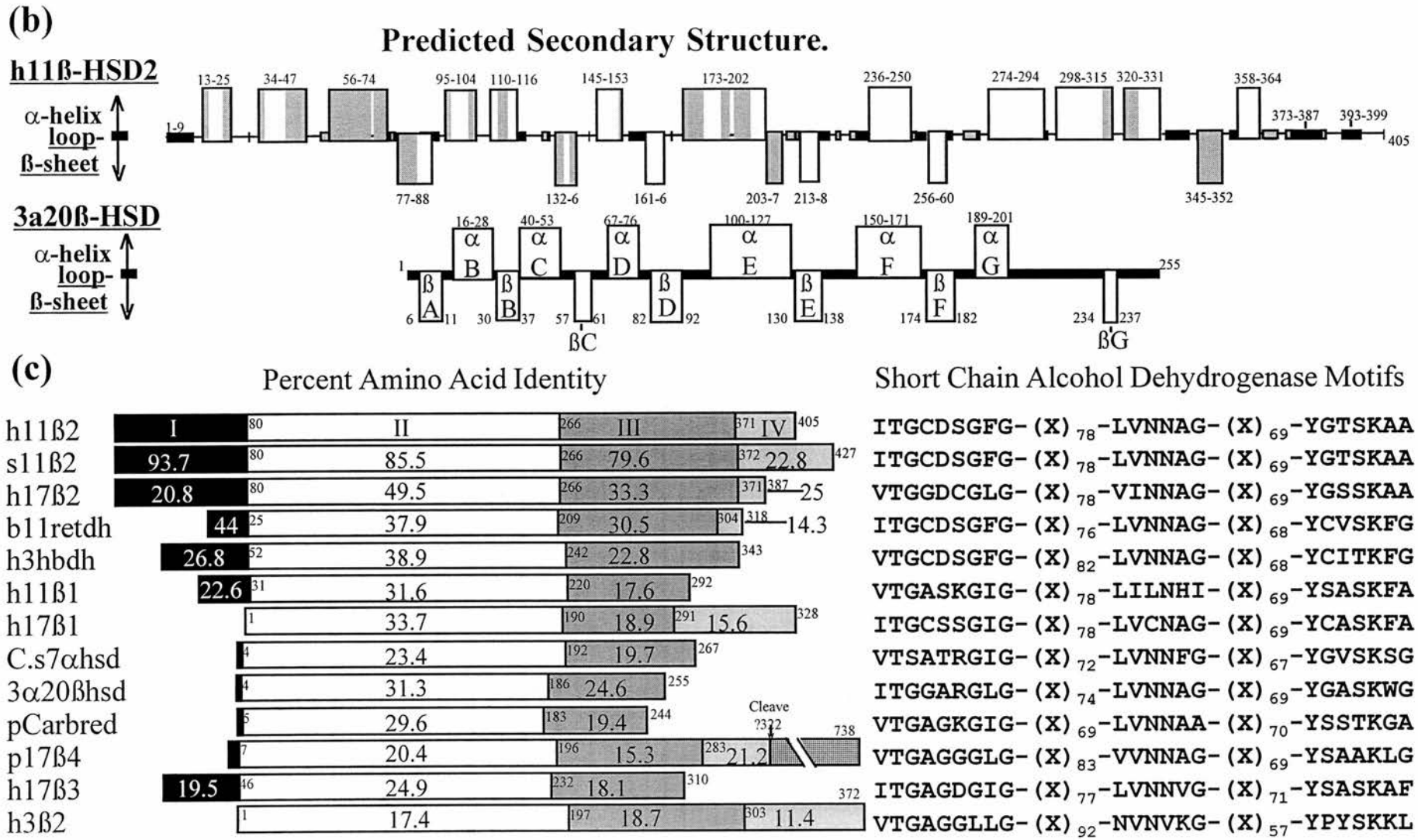


Fig 7.2.b/c (see previous page for legend).

predicted net positive charge (+19) and very basic predicted isoelectric point of 9.92 for the 11ß-HSD2 polypeptide. Nine cysteine residues are present, one of which occupies the position at which a 'blank cycle' resulted on sequencing of an 11ß-HSD2 peptide (cycle 15, peptide C, see Chapter 6) suggesting this residue may be particularly reactive or modified in the native protein.

7.2.2.b Detailed analysis of the predicted structure of the 11ß-HSD2 protein.

Analysis of the primary (fig.7.2(a)) and predicted secondary structure(fig7.2(b)) of the 11ß-HSD2 protein suggest four distinct regions. The most N-terminal region (region I) begins with an exposed loop (Met1-Gly9) followed by a very leucine-alanine rich area (60% of residues 11-73). This has some incomplete repeats (AALALLAAL, residues 36-44, close variants beginning at residues 16 and 56) and appears likely to have secondary structure broken by helix-breaking residues (Gly31 + Pro33, Pro53-Pro54-Pro55) into 3 buried, predicted -helical, segments centring on residues 16-22, 35-41 and 63-69 respectively. The region has imperfect heptad symmetry, and in such a secondary structure is likely to form a particularly hydrophobic face along which leucines align, there being 2 such potential axes:- (1) L14-L21-L28-L35-A42, stretching around to the nearby helical face L13-L20-D27-L34-L41 and (2) reaching further C-terminal in the region L23-L30-A37-L44-L51-L58-G65-L72. On the helical aspect opposite to these axes all 7 positively charged residues in the area line-up: R18-R25-R32-----R74 and R29--R50--R71. Such a structure is suggestive of a domain with a leucine zipper-like tendency to form protein-protein interactions (often dimerisation) along the hydrophobic axes.
The second region (region II), which unlike the rest of the protein has predicted secondary structure rich in ß-sheets, contains 3 motifs characteristic of members of the SCAD superfamily. Firstly, the putative cofactor binding site motif (V/I)TGXXXGXG, is present (ITGCDSGFG, residues 87-95) in an area with secondary structure predicted to incorporate buried ß-sheet(84-88), buried loop(89-92) and an exposed helix(96-102). Secondly a "substrate positioning" motif, noted in previous alignments of SCAD enzymes[42], which is of the form [LFVM][VI][NL][N][AVH(F)][GI] is present (LVNNAG, residues 164-169). The major predicted features of this substrate positioning area are an exposed loop (156-160)/buried ß-sheet(161-166+) being preceded by an -helix (centred on residues 145-152) and exposed flexible region likely to loop around Pro142-Gly143. Thirdly, the putative catalytic motif YXXXKX[SAG] is present (YGTSKAA, residues 232-238). This motif, which is predicted to form a small loop around Gly233, is in the centre of a stretch predicted to have flanking areas consisting of loop buried ß-sheet loop structures (at 211-223 and 252-264). A long hydrophobic -

helix (residues 236-250) bridges the motif-loop/sheet/loop stretch to the C-terminal side, however the secondary structure is unclear for the region bridging (residues 224-231) between the motif and the loop/sheet/loop on the N terminal side. The equivalent of this 'upstream bridging region' in the related SCAD enzyme 3 20ß-HSD was shown to be part of the steroid substrate (cortisone) binding pocket in Xray crystallographic studies[55].

Region III, residues 275-370, has three positively charged segments, EKRKQ, ARPRRRY, and LRRRF (residues 277-281, 332-338 and 358-362 respectively). Such segments may form charge interactions with negatively charged molecules or exert electrostatic influence on a substrate. In the context of a membrane protein, interactions with phospholipids helping to stabilise or anchor the protein are particularly likely and often occur adjacent to helical transmembrane domains (e.g. glycophorin[173]). Indeed most of the region between the charged segments has predicted helical secondary structure, especially residues 274-294, 298-312 and 324-331).

Finally, region IV, the C-terminal region, is proline/glycine rich (36% of residues 373-405) and is predicted to form a flexible area with several exposed loops. It contains the only potential N-glycosylation site in 11ß-HSD2. The potential for glycosylation at this motif is weakened as it is flanked by prolines. Moreover amino acid sequencing yields across the B-peptide (fig.7.2) suggested Asn394 was largely unglycosylated in the native protein from human placenta (see Chapter 6).

Although much of the secondary structure can be predicted with some certainty there are important aspects of the higher order structure of 11ß-HSD2 that are unclear. As 11ß-HSD2 is an intrinsic membrane protein from a family of enzymes (SCADs) thought usually to be tetrameric in the native form, it probably contains both transmembrane segments and dimerisation interfaces. The N-terminal leucine-alanine rich region (residues 11-73) may potentially fulfil either role while the helical/charge cluster region (residues 275-370) contains predicted hydrophobic helical segments which are good candidates as possible transmembrane regions. Dimerising elements outside the N-terminal region seem likely, as a number of other SCADs which lack such a region have been demonstrated to form tetramers. Fig.7.2(b) also illustrates the secondary structure of the related SCAD enzyme 3 20ß-HSD as determined from x-ray crystallography studies. There is a very striking similarity within region II (the SCAD region) with almost all the major structural features from ßA-ßF of 3 20ß-HSD being represented clearly in the predicted 11ß-HSD2 structure (the only exception being that ßB corresponds to a segment of 11ß-HSD2 (residues 104-109) with uncertain secondary structure). Studies on 3 20ß-HSD have identified areas involved in steroid and cofactor binding and in dimerisation; some of the corresponding regions in 11ß-HSD2 may have similar

functions The dimerisation interfaces of 3 20ß-HSD include the region between the LVNNAG and YXXXK SCAD motifs (E-ßE- F) and the most C-terminal ß-sheet element (ßG) [55].

7.2.2.c Sequence Similarities to Human Placental 11ß-HSD2.

A search of sequence databases shows the most clearly related proteins are SCAD members (fig.7.2(c)), the closest being microsomal NAD-dependent human 17ß-HSD type2[62] (38.9% amino acid identity), retinal pigment epithelium NAD-dependent bovine 11-cis retinol dehydrogenase (35.5% identity) and the mitochondrial human NAD-dependent enzyme 3-hydroxybutyrate dehydrogenase[56] (32.6% identity). There is 28.3% identity to human 11ß-HSD type1[41] (microsomal, NADP-dependent). Similarity of the four 11ß-HSD2 regions (I-IV) are shown in fig.7.2(c) for these enzymes and others with some functional similarity that are related to 11ß-HSD2. The three SCAD motifs referred to above are highly conserved although the middle motif is atypical in 11ß-HSD1 and shifted in relative position or absent in 3ß-HSD2 (fig.7.2(c)). This latter enzyme appears to have the other 2 SCAD motifs in the same orientation and with spacing typical of SCADs. This highlights the fact that the bi-functional 3ß-HSD family (which share high sequence identity and also have ketosteroid isomerase activity) fit somewhat uncomfortably into the SCAD superfamily and it is unclear whether they should be considered as SCADs or as a separate family. Very recently NAD-dependent 11ß-HSD enzymes have been expression cloned from kidney in sheep[68] and human[70]. The sheep kidney 11ß-HSD2 has 78.5% identity at the protein level (82.6% at nucleic acid level). Similarity is non-uniform across regions I-IV at both protein(fig.7.2(c)) and nucleic acid levels; thus sheep 11ß-HSD2 has 94.2%, 88%, 80% and 65% identity to human 11ß-HSD2 at the cDNA level in regions I-IV respectively. The 5'UTR and most of the 3'UTR have 70% identity, however this rises sharply again between bases 1536-1624, fig.7.2(a). (91% identity, reason unclear) and for sequences adjacent to the polyadenylation motif aaataa (92%: bases 1731-1764: fig.7.2). The human renal clone is similar, but not identical, to the cDNA from placenta reported here. The renal clone lacks the first 25 bases (-133 -109), has 2 deletions in the 3'UTR (bases 1270 and 1495: fig.7.2) and a base substitution at 442 resulting in a change in the predicted amino acid sequence, $148^{Val\ \ Leu}$. At the points of difference, the sequence reported above has been confirmed in cDNA we have sequenced derived from a second placenta, and the Val148 residue is clearly present in human placental 11ß-HSD2 as it is the first amino acid of the D peptide sequence derived from purified 11ß-HSD2 tryptic digests (Table 6.1). Although it is most likely these differences arise from

polymorphisms, there may be isoform microheterogeneity similar to that observed with other steroid metabolising enzymes notably 3 -HSD (98.8% identity) [43,114] and 3ß-HSD (rat 3ß-HSD2 being 99.2% and >93% identical to 3ß-HSD2 male liver variant[53] and 3ß-HSD1[115] respectively). The finding of polymorphisms that affect the 11ß-HSD2 protein sequence is clearly of considerable interest, especially if a difference in steroid metabolism is associated. Placental 11ß-HSD2 has a K_m for corticosterone (see Table 8.1) virtually identical to that of the 1000-fold purified native 11ß-HSD2 from placenta (see Chapter 4). The K_m reported for the renal clone is 3-fold lower (4.4nM) and it could be that amino acid substitutions at residue 148 cause subtle alterations in steroid metabolising activity by affecting the predicted -helical region of which it is part.

Chapter 8 : Expression of Recombinant 11ß-HSD2 in Cell Free Lysates and Mammalian Cells.

8.1 Introduction.

Analysis of the predicted amino acid sequence of the p11ß2 clone revealed that it encoded for an enzyme of the SCAD superfamily which incorporated all the 11ß-HSD2 tryptic peptide sequences (see Fig.7.2). However to finally prove that expression of the mRNA corresponding to the p11ß2 cDNA was necessary and sufficient to produce both the 11ß-HSD2 activity characterised in human placenta (see Chapter 4) and the ≈40kDa protein which could be affinity labelled (see Chapter 5), it was necessary to demonstrate that these properties were conferred upon mammalian cells when transfected with the p11ß2 cDNA. Other members of the SCAD superfamily have extensive post-translational modifications, for example 17ß-HSD type 4[116]. Some indication of the extent of post-translational modification could be obtained by examining the protein products of expression of the 11ß-HSD2 cDNA in coupled *in vitro* transcription/translation systems.

8.2 Results.

8.2.1 Expression Studies with Human Placental 11ß-HSD2 cDNA.

8.2.1a Expression of 11ß-HSD2 protein.

Expression of p11ß2 in CHO cells did indeed produce abundant 11ß-HSD2 enzyme activity (see below), accompanied by the expression of a 40k protein which could be affinity labelled with corticosterone (fig.8.2, lanes 2 cf. 1 and 10 cf. 11), in a similar manner to affinity labelling of 11ß-HSD2 from crude placental sub-cellular fractions (fig.8.2 lanes 3 and 9). Coupled *in vitro* transcription-translation of 11ß-HSD2 in rabbit reticulocyte lysates (without microsomal processing) also produced a protein (fig.8.2, lanes 4 and 8) of similar size to 11ß-HSD2 in placenta or expressed in CHO cells. Thus activity in these tissues appears not to require major cleavages or large covalent attachments. Addition of canine pancreatic microsomes resulted in a small size increase and broadening of the protein band, suggesting co-translational processing, possibly involving glycosylation, was occurring (fig.8.2 lanes 4-8). Addition of increasing amounts of microsomes also resulted in a reduced efficiency of translation (a standard finding with TNT® lysates) and accordingly more sample was loaded (to facilitate size

comparison), and a minor band at 31k became visible. It is unclear if this is a different, minor, translation product or the result of 11ß-HSD2 proteolytic cleavage (from processing or degradation) occurring at low level in the presence of these microsomes. Clearly a range of processed states of 11ß-HSD2 is demonstrated and may indicate the possibility of tissue specific co-translational processing depending on the activities within host tissue microsomes. If cleavage to a 31k form occurs, this is likely to affect enzyme structure/function and possibly its subcellular localisation. Finally although native placental 11ß-HSD2 is most likely to be located in microsomes this is not confirmed by the presence of a classical C-terminal microsomal retention motif[117,172], in contrast to the closely related human 17ß-HSD2[62].

8.2.1b Characteristics of Expressed Enzyme Activity.

Expression of the 11ß-HSD2 cDNA in CHO cells produced, high affinity 11ß-HSD activity which had the expected characteristics of 11ß-HSD2 activity in placenta. This was exclusively NAD-dependent in cell homogenates (12nM corticosterone and 0.15mg protein/ml), showing 49% conversion with 400µM NAD, whilst 400µM NADP produced no increase over assay with no added cofactor (1.8%), see fig.8.1 (lanes 1-3). No 11ß-reductase activity was detected in homogenates of 11ß-HSD2-transfected CHO cells, or in the medium of the intact cells (which metabolised 99% of 25nM ^{3}H-corticosterone in 24 h: fig.8.1, lane 4). 11-dehydro products were the only metabolites detected by HPLC. Thus after 1 h incubation of ^{3}H-steroid (12nM) with recombinant 11ß-HSD2 (0.5mg homogenate protein/ml), >95% of corticosterone, 63% of cortisol and 17% of dexamethasone were metabolised, whilst aldosterone was unaltered (Fig.8.1, lanes 6-9). There was no evidence of 5 or 5ß reductase activity encoded by p11ß2.
Recombinant human placental 11ß-HSD2 has a high affinity for glucocorticoid substrates, especially corticosterone (Table 8.1). At corticosterone concentrations higher than 80nM there appeared to be a degree of product/substrate inhibition. Examination of 11ß-HSD2 inhibition by a range of steroids showed potent inhibition (=IC_{50}) by glycyrrhetinic acid (11nM) and carbenoxolone (18nM), with significant inhibition by 11-dehydro corticosterone (45nM), progesterone (0.8µM) and cortisone (1µM), whilst there was little effect (>30µM) with DHEAS, DHEA, testosterone and estradiol (fig.8.3). Affinity labelling studies on transfected CHO cells, as with similar studies on unpurified 11ß-HSD2 in placental fractions (Chapter 5: fig.5.1), revealed the same rank order of potency in labelling (corticosterone >> cortisol >> dexamethasone, aldosterone = nil) as indicated by K_ms (Table 8.1) for affinity as 11ß-HSD2 substrates. A rank order of affinity

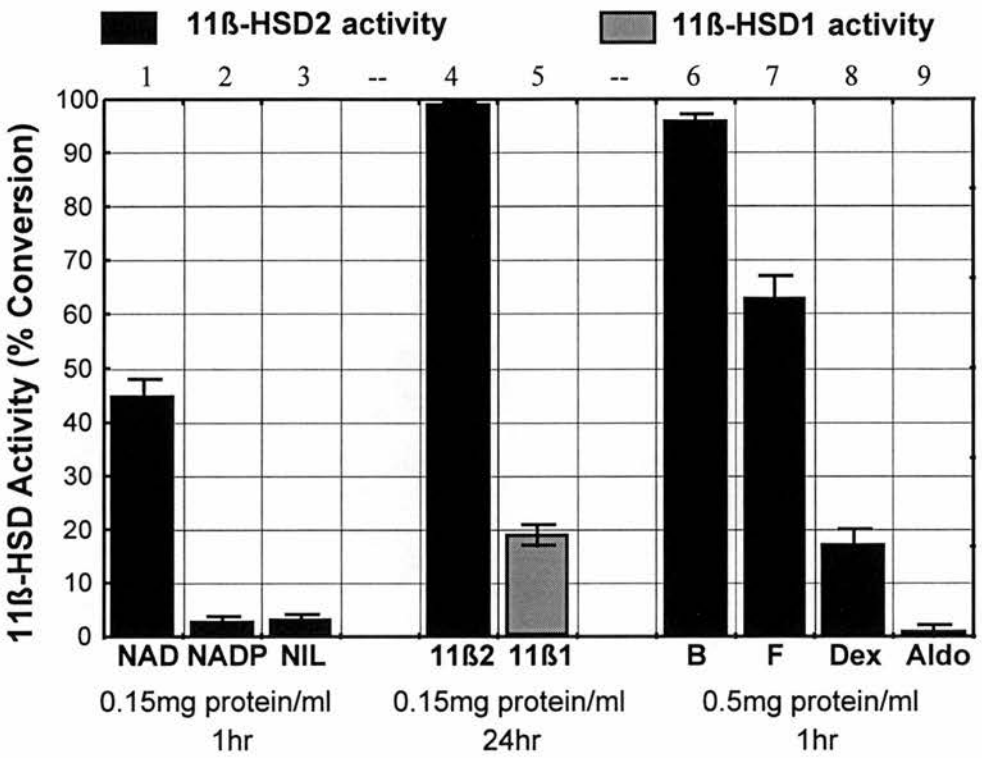


Fig. 8.1 Enzyme activity produced by transient transfection of 11ß-HSD2 cDNA in CHO cells. CHO cells transfected with 11ß-HSD2 cDNA (p11ß2 clone, **lanes 1-4, 6-9**) were homogenised and assayed [mean ± SE (n=3) 11ß-dehydrogenase] at the protein concentrations and for the assay durations *indicated below the columns*. The results indicate the enzyme activity encoded by the 11ß-HSD2 cDNA is NAD-dependent (**lanes 1-3**), metabolises glucocorticoids [(corticosterone(B) > cortisol(F) > dexamethasone(Dex), **lanes 6-8**] but not aldosterone (**lane 9**) and is overwhelmingly dehydrogenase, going to completion (>99%, **lane 4**) in contrast to the reversible 11ß-HSD1 activity from homogenates of CHO cells transfected with the rat 11ß-HSD1 cDNA (**lane 5**, ≈ 20% dehydrogenase conversion).

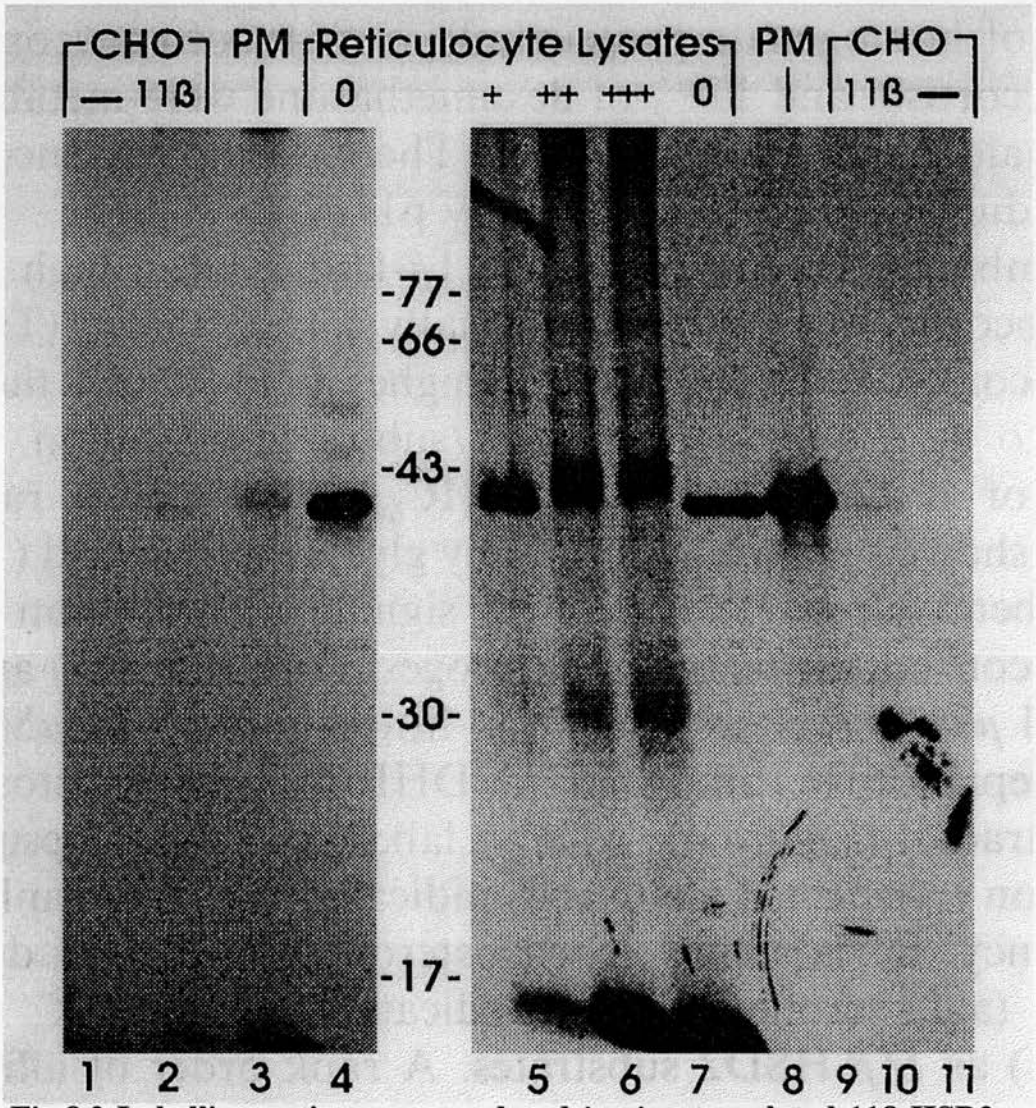


Fig.8.2 Labelling native, expressed and *in vitro* translated 11ß-HSD2 protein. Lanes 1-3 + 9-11. Affinity labelling with ^{3}H-corticosterone of (PM) native human placental 11ß-HSD2 from placental membrane fractions (lanes 3 and 9): 20 and 80µg protein loaded respectively, from 25000g pellet (heavy microsomal and mitochondrial fraction)) and (CHO) homogenates of CHO cells (160mg protein/lane) transfected with 11ß-HSD2 cDNA (lanes 2 and 10) and with vector only (lanes 1 and 11). **Lanes 4-8**, products of coupled *in vitro* transcription-translation of 11ß-HSD2 cDNA (sub-cloned into pGEM11zf) in rabbit reticulocyte lysates labelled with ^{35}S-methionine in the presence of varying amounts of microsomes (*indicated above lane*: 0, + = 1.5µl, ++ = 3µl, +++ = 4.5µl (excess)). Lysate volume loaded in lanes 4-8 was 0.5µl, 1µl, 1µl, 1µl and 0.1µl respectively. The volume loaded was varied to facilitate size comparison of the translated products and was needed to counter the reduction of translation in presence of higher microsome concentrations. Position of protein standards (M_r x 1000) is indicated.

Glucocorticoid.	K_m (nM)	$V_{max.}$ (pmol/[h,mg protein])
Corticosterone	12.4±1.5	8.0 ± 0.7
Cortisol	43.9±8.5	4.8 ± 0.7
Dexamethasone	119±15	3.7 ± 0.5
Aldosterone	not metabolised	-----

Table.8.1 Kinetic parameters of 11ß-HSD2 activity in cells transfected with 11ß-HSD2 cDNA. All estimates based on at least 10 steroid concentrations, n = 5 at each concentration for corticosterone, n=2 for cortisol and dexamethasone.

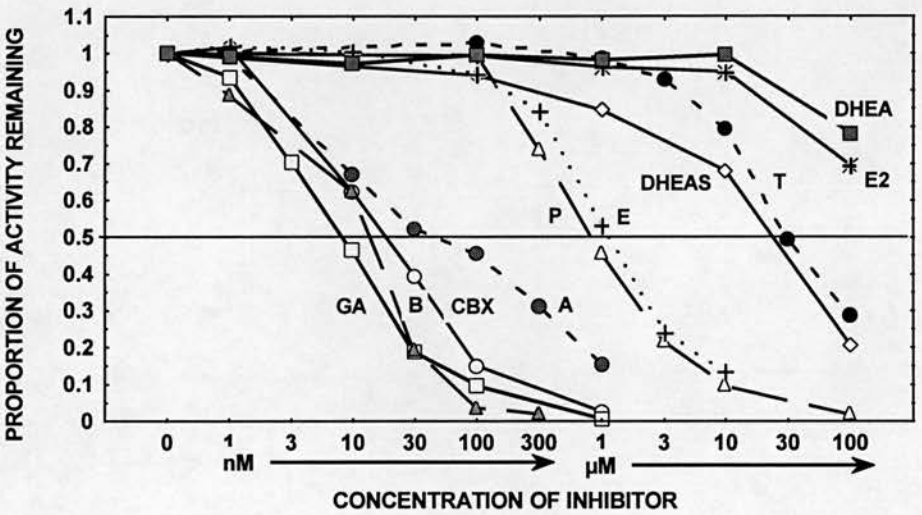


Fig.8.3 Studies on inhibition of 11ß-HSD2. The extent to which a range of compounds reduce recombinant 11ß-HSD2 conversion of 12nM ^{3}H-corticosterone is shown. GA = glycyrrhetinic acid, CBX = carbenoxolone, A = 11-dehydrocorticosterone, P = progesterone, E = cortisone, E2 = estradiol, DHEA(S) = dehydroepiandrosterone (sulphate). Reactions in presence of 400µM NAD, using homogenates (0.15mg protein/ml) of cells transfected with 11ß-HSD2 cDNA. N=4, with standard errors being < 4%.

as substrate/inhibitor is thus suggested for placental 11ß-HSD2 of: glycyrrhetinic acid carbenoxolone corticosterone > 11-dehydro corticosterone cortisol > dexamethasone > progesterone cortisone >> DHEAS, testosterone, estradiol, DHEA, aldosterone. This rank order is very similar to that determined for the type 3 'corticosterone' receptor sites[22,98] and suggests these 'sites' are related to 11ß-HSD2, but whether they are actually due to the 11ß-HSD2 protein remains unclear. In late human gestation progesterone levels often rise to 0.3-0.5μM in the mother and >1μM in the fetus[113]. Thus the IC_{50} for 11ß-HSD2 of progesterone (0.8μM) may signify a potential influence of progesterone on corticosteroid action during late pregnancy.

8.3 Discussion.

The p11ß2 clone has all the expected characteristics of the cDNA encoding human placental 11ß-HSD2. Thus, expression of the p11ß2 cDNA in mammalian cells produced 11ß-HSD2 enzyme activity and concurrent appearance of a ≈40kDa protein which could be affinity labelled by the technique used in the purification of the 11ß-HSD2 protein.

The p11ß2 cDNA produced a protein band of ≈40kDa as the only significant product of *in vitro* transcription/translation without co-translational processing. However on addition of increasing amounts of microsomes, co-translational processing gave rise to a number of products very close to 40kDa in size. It was clear from the purification of 11ß-HSD2 from placenta that a number of products ≈40kDa (see fig.6.1) co-purified with the 11ß-HSD2 activity across AMP affinity chromatography (implying they may be related, possibly being processing variants retaining NAD-binding capacity at least), and ran nearby to affinity labelled 11ß-HSD2 on 2-D electrophoresis (see fig.6.2). Thus, there is some evidence to suspect 11ß-HSD2 processing variants akin to those seen at ≈40kDa at *in vitro* translation may exist in human placenta. There is no good evidence for a 31kDa band co-purifying from human placenta, but such a drastic change in structure as loss of 10kDa would be expected to affect substrate affinity and thus also binding during AMP affinity chromatography. In Chapter 10 (fig.10.3), evidence is presented of a ≈31kDa band in human salivary gland detected on Western blots using specific antiserum raised to the C-terminus of 11ß-HSD2 in addition to the ≈40kDa band which is the only band seen in human placenta. This raises the possibility that a product similar to the one described above on *in vitro* translation with excess pancreatic microsomes is produced in a tissue specific manner. The function of such a putative 11ß-HSD2-related variant is currently unclear.

Chapter 9 : Generation and Characterisation of Polyclonal Anti-11ß-HSD2 Antiserum.

9.1 Introduction.

No antisera to the 11ß-HSD2 enzyme from any species were available and thus the determination of human placental 11ß-HSD2 amino acid and cDNA sequences brought the prospect of raising specific anti-11ß-HSD2 antiserum for the first time.

9.2 Method.

The 11ß-HSD2 peptide sequences and adjoining amino acid sequence from the cDNA were inspected to choose a suitable region to use in a peptide-carrier conjugate for generation of antisera. It is known that the C-terminus of a protein is, in general, disproportionately antigenic and so keeping the chosen region near the C-terminus was an advantage. However, minor cleavages and modifications to proteins are commoner in their terminal portions and a consensus glycosylation sequence (NLS, residues 394-6) was present in the B-peptide only 8 residues from its C-terminus (12 residues from the 11ß-HSD2 C-terminus). Although this residue seemed unmodified in placenta (see Chapters 6 and 7) it was decided to not cross this site in the chosen peptide. Accordingly, a 14 amino acid region HCLPRALQPGQPGT which had high predicted flexibility and was slightly further from the C-terminus (11ß-HSD2 residues 370-383, see figs.9.1 and 7.2), was chosen as the 11ß-HSD2 peptide for raising specific antibody and was produced by solid phase synthesis[§].

As the 11ß-HSD2 peptide was near C-terminal it was expected to be more antigenic if coupled at the N-terminus, so leaving its C-terminus free. There were two options for such coupling:- firstly to couple via the near N-terminal cysteine or secondly to couple via the histidine at the N-terminus. To increase the chances of success both options were used making two separate conjugates (C- and N-conjugates , respectively) to key-hole limpet haemocyanin at peptide: carrier ratio of 50:1[‡].

Three rabbits (adult New Zealand White) were immunised with each conjugate, inoculated initially in Freund's complete adjuvant and boosting monthly in Freund's incomplete adjuvant.

Site within 11ß-HSD2 protein of Targeted Peptide.

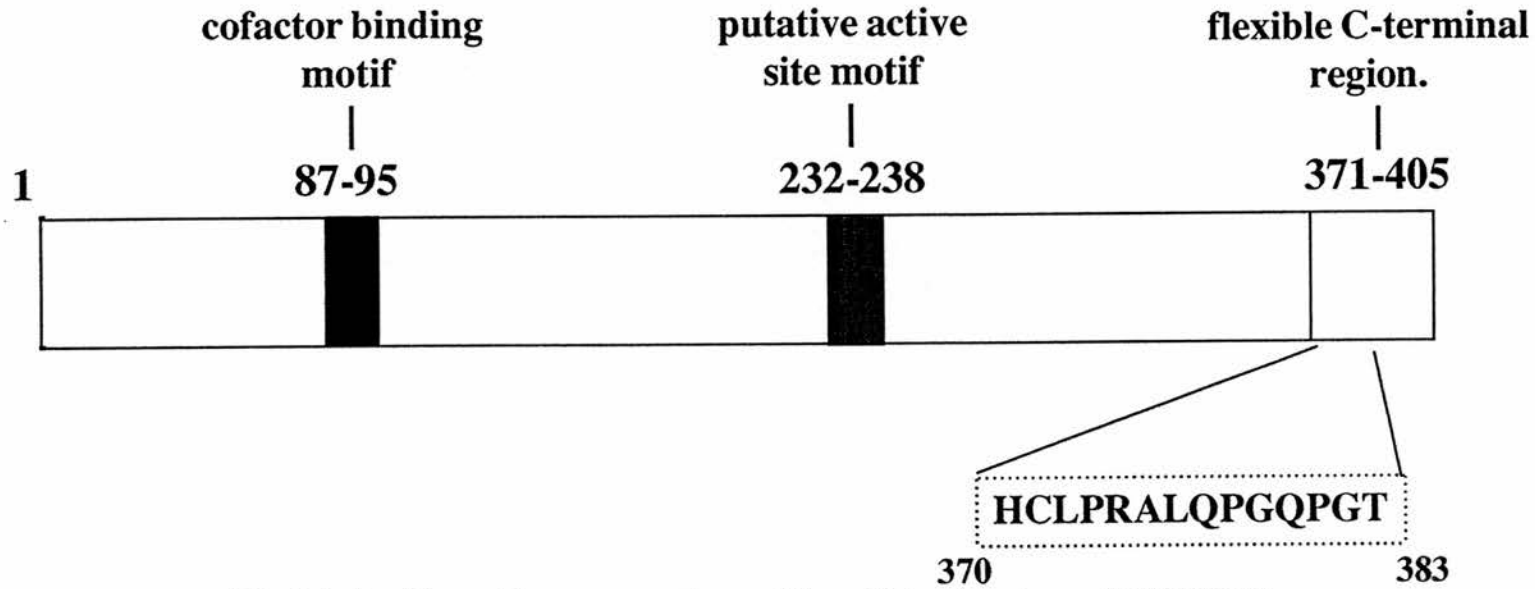


Fig.9.1. Position of immunogenic peptide within structure of 11ß-HSD2 protein. 11ß-HSD2 consists of 405 amino acids, the final C terminal region (amino acids 365-405) is predicted to have a flexible structure. A 14 amino acid peptide from this region (amino acids 370-383) was synthesised and coupled to keyhole limpet haemocyanin and rabbits were inoculated with the conjugate in Freund's adjuvant.

9.3 Results and Discussion.

The antisera collected during the immunisation protocol were tested for the ability to detect 11ß-HSD2 protein on strips cut from a Western blot of human placental proteins processed with antisera from rabbits immunised with the 11ß-HSD2 conjugates. It can be seen from fig.9.2 that by the third boost ('C-series' of antisera) rabbit 5 produced a high titre antiserum recognising a 40kDa protein and that the strength of this specific reaction at 40kDa had risen dramatically compared to the antiserum from the same rabbit after the previous boost as shown in lane 5B. The titre of this antibody continued to rise further after the fourth boost (5D) as the strong band in lane 2 of fig9.3 shows(using 5D at $1/10^4$). Fig9.3 also shows the band has the expected distribution of 11ß-HSD2 amongst fractions eluting from AMP affinity chromatography. Fig.9.4 shows that the ≈40kDa band appears on transfection of CHO cells with the 11ß-HSD2 (p11ß2) cDNA and detects a band the same size in kidney. A minor band at ≈70kDa is also sometimes seen in kidney fractions, the significance of this is currently unknown.

Thus the 5C/D antiserum specifically detect a protein(≈40k) with all the following characteristics expected of the 11ß-HSD2 enzyme protein and does so very clearly at up to 1/10000 dilution:-

1) The detected protein appears on transfection of mammalian (CHO) cells with the p11ß2 cDNA, co-incident with 11ß-HSD2 enzyme activity.

2) The protein is ≈40k, the size of 11ß-HSD2 purified from placenta.

3) Like 11ß-HSD2 it binds to and is purified by N6-linked 5'AMP-agarose and is absent from placental subcellular fractions lacking 11ß-HSD activity i.e. cytosol (lanes 3 and 7 fig.9.3)

4) It is present in placenta and kidney and as is shown on further tissue localisation work (Chapter10: fig.10.3) to be absent from a range of tissues lacking 11ß-HSD2 enzyme activity.

Moreover, the antiserum not only has a high titre of anti-11ß-HSD2 antibody but the 40kDa protein is the major (or only significant band) detected in placental or kidney tissue. Thus this 5C/D antibody seems to have the properties required to be very useful in the study of 11ß-HSD2 in human tissues both on western blots and immunohistochemistry. There was also the unusual advantage, for a polyclonal antibody, that the specificity of positive immunostaining could be validated not only by pre-immune serum controls but also by pre-absorbing the 5C/D antiserum with excess 11ß-$HSD2_{370\text{-}383}$ peptide so sequestering the anti-11ß-HSD2 antibodies. Results with such immunostaining, with and without peptide preabsorption, are presented in Chapter 10.

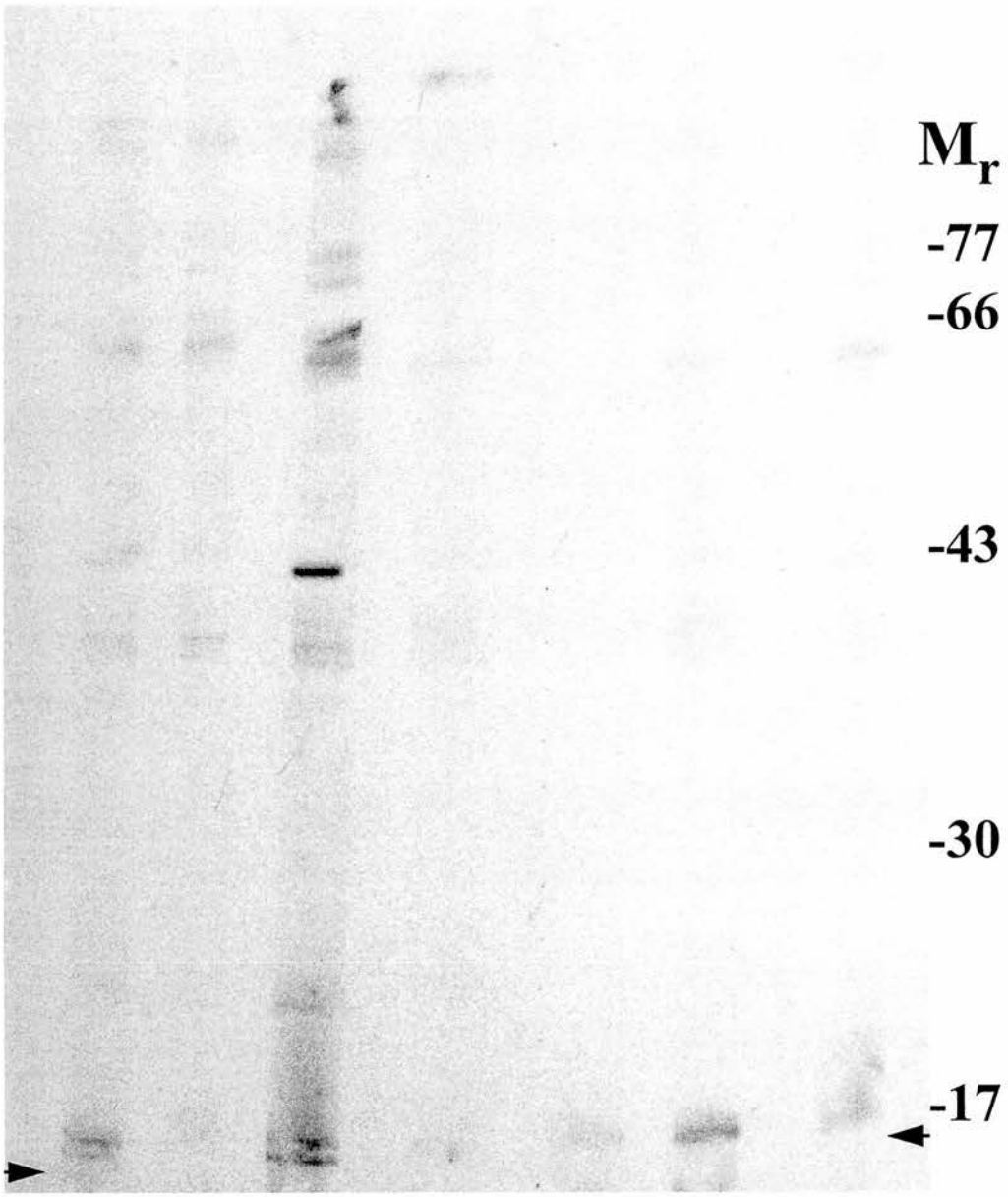


Fig.9.2 Raising of 11ß-HSD2 peptide370-383 antisera. Human placental western blot analysis of antisera from 6 rabbits after the third innoculation (lanes 1C-6C) of 11ß-HSD2 conjugate. Lane 5B is comparison from second innoculation of rabbit 5. Position of dye front (arrow heads) and protein standards are indicated (M_r/1000).

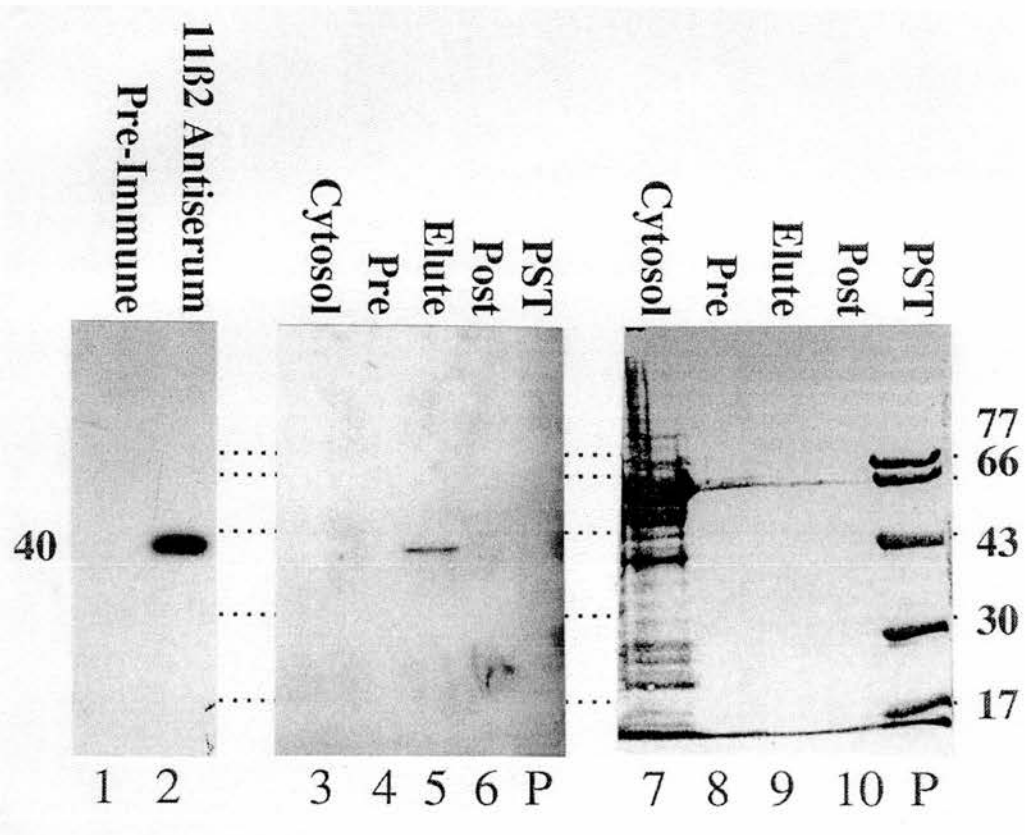


Fig.9.3. Confirmation that antiserum detects band co-segregating with 11ß-HSD2 activity. (*Left panel*) Western blots (Amersham ECL) of subcellular fractions of human placenta with high 11ß-HSD2 activity (25000g pellet, heavy microsomes/ mitochondria) using: lane1 pre-immune antisera (at 1/100 dilution), lane 2 11ß-HSD2 antisera (at $1/10^4$). (*Middle panel*) Western blot using anti-peptide370-383 antisera. Lane 3, human placental cytosol (11ß-HSD2 activity ≈ nil); lanes 4-6, fractions from 5'AMP affinity chromatography (purifying 11ß-HSD2 >1000-fold) before (lane 4), during (lane 5) and after (lane 6) elution of 11ß-HSD2 activity respectively. (*Right panel*) Ponceau stain of membrane used in the middle panel, before detecting with antibody. PST = protein standards (M_r indicated *on right*).

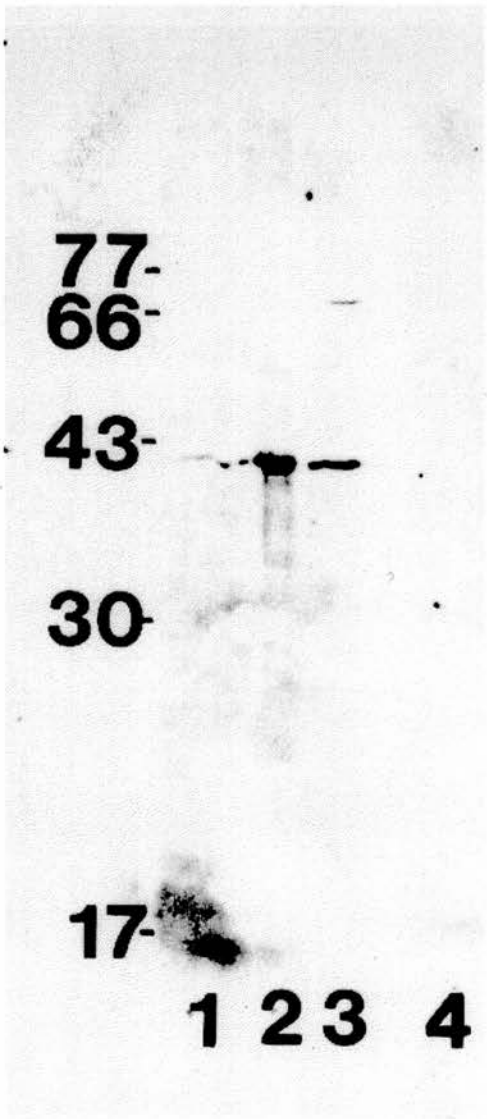


Fig.9.4 Proof the antibody raised detect protein encoded by 11ß-HSD2 cDNA. Western blots using anti-11ß-HSD2 antisera on 25000g pellet fractions (heavy microsomes/ mitochondria) of human placenta (lane2), human kidney (lane3) and CHO cells transfected with (lane 1) and without (lane 4) human placental 11ß-HSD2 cDNA. Position of protein molecular weight markers (M_r/1000) indicated on left.

§ Solid phase peptide synthesis was carried out by Dr Bala Ramesh, Royal Free Hospital School of Medicine, London.

‡ Peptide-carrier conjugation was carried out by Dr Brian McGinn, Thistle Peptide Services, University of Glasgow.

Chapter 10 : Tissue Distribution of 11ß-HSD2.

10.1 Introduction.

11ß-HSD2 plays a crucial role in controlling glucocorticoid action and mineralocorticoid receptor specificity and so its tissue distribution was of great interest. Having cloned the 11ß-HSD2 cDNA (Chapter 7) and raised a specific anti-11ß-HSD2 antibody (Chapter 9) it was now possible to determine distribution of 11ß-HSD2 gene expression and to look in detail at the expression in key tissues. This distribution was examined by using northern blots of human adult and fetal tissues and using *in situ* hybridisation to look at the detailed distribution of 11ß-HSD2 mRNA in placenta and kidney. Investigation of the detailed distribution of the 11ß-HSD2 enzyme protein in these two key tissues was also determined using the specific anti-human 11ß-HSD2 antibody raised as described in Chapter 9.

10.2 Results.

10.2.1 Distribution of 11ß-HSD2 mRNA in human tissues.

10.2.1a Northern blot analysis.

Northern analysis revealed a hybridising 1.9kb transcript in placenta, aldosterone target tissues (kidney, colon, parotid, skin) and pancreas and fetal kidney (fig.10.1). There was also hybridisation to mRNA of a larger size (4kb) in fetal kidney. Under these conditions there was no detectable hybridisation to RNA from liver, oesophagus, stomach, ovary, breast, fetal lung, fetal liver or fetal brain nor to adult human brain sub-regions (frontal cortex, cerebellum, hippocampus, hypothalamus, pons and medulla: data not shown).

10.2.1b In situ hybridisation.

In situ hybridisation, with 11ß-HSD2 cDNA, on normal human adult kidney (fig.10.2) showed abundant 11ß-HSD2 mRNA expression restricted to the distal nephron (distal convoluted tubule, cortical collecting duct and medullary collecting ducts) with no specific hybridisation in other regions (glomerulus, proximal tubule etc.). Expression in distal convoluted tubule extended to loops participating in juxtaglomerular complexes. There was no hybridisation in sense controls. There was also expression of 11ß-HSD2 mRNA in human placental syncytiotrophoblast.

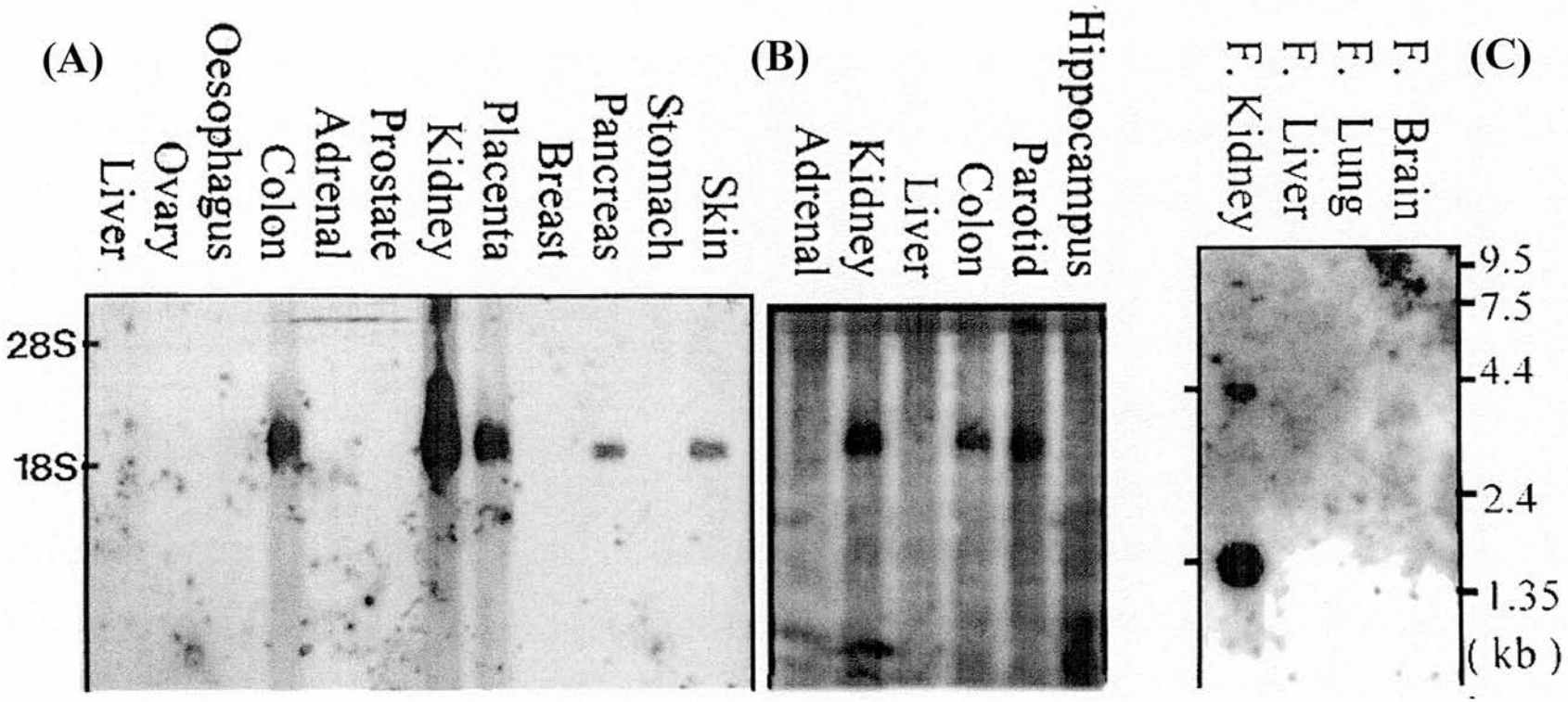


Fig.10.1. Northern hybridisation of 11ß-HSD2 cDNA in human tissues. Hybridisation of RNA from a range of (**panels A + B**) human adult (total RNA) and (**panel C**) fetal (highly purified polyA RNA) tissues with random-primed human placental 11ß-HSD2 cDNA probe (bases 217-1737: fig7.2). Position of fetal RNA markers and 18S and 28S indicated. Check-marks on left of fetal blot correspond to level of the two mRNA species hybridising : at 1.9 and ≈4kb.

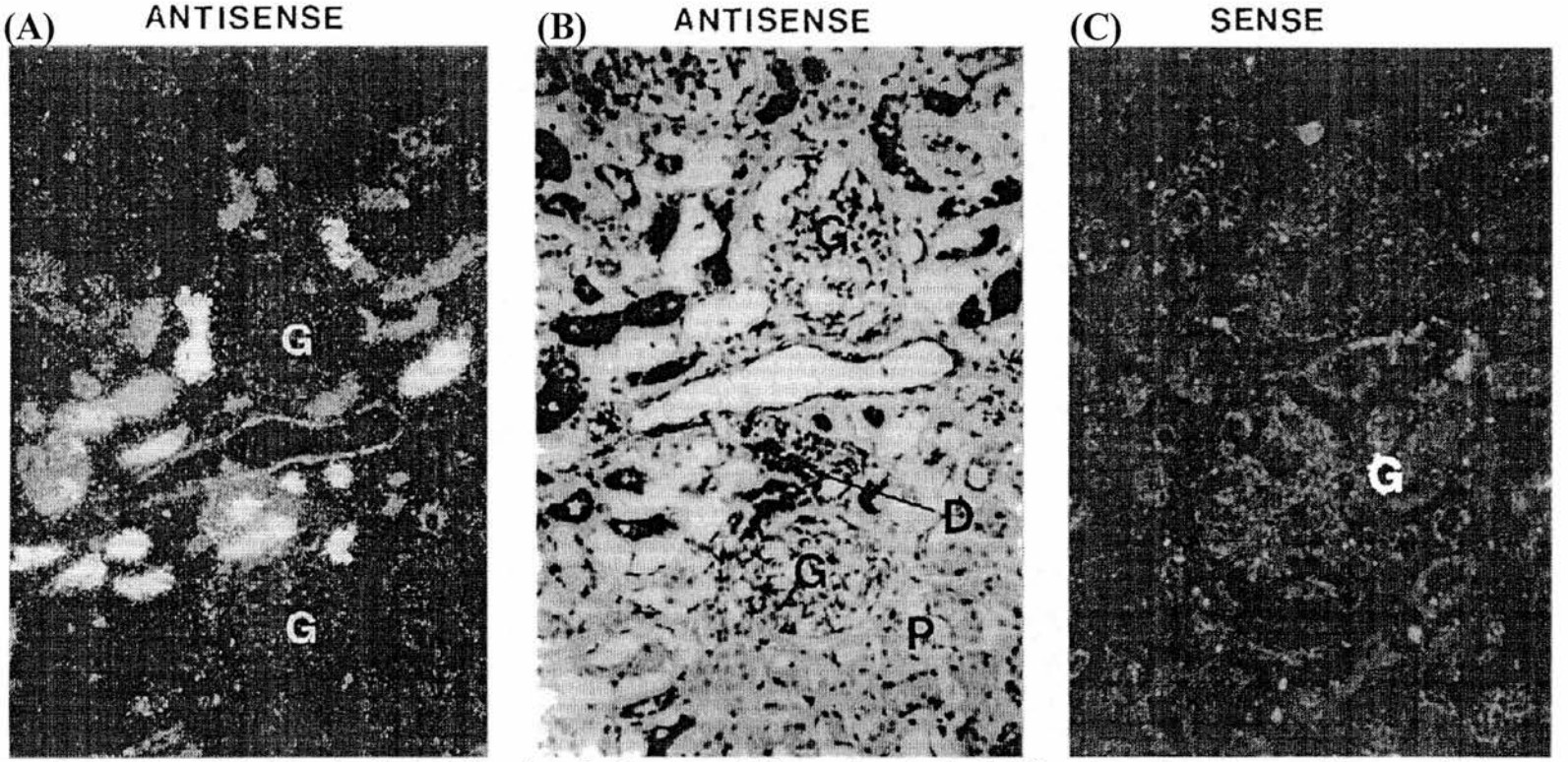


Fig.10.2 11ß-HSD2 *in situ* hybridisation in human kidney. *In situ* hybridisation of sections of human kidney to human placental 11ß-HSD2 cRNA probes (sense and antisense to CtBb fragment, dashed box fig.7.2) **Panel (B)** = light field (silver grains = dark), whilst **panels (A + C)** are dark field (silver grains = white). **Panels (A + B)** are of the same section, **(B)** at slightly higher magnification, whilst **panel (C)** is of a neighbouring section from the same kidney slice (at higher magnification) The following are labelled: G, glomerulus; D, distal convoluted tubule; P, area with many proximal convoluted tubules. Clearly 11ß-HSD2 mRNA is abundant in the distal nephron.

10.2.2 Distribution of 11ß-HSD2 protein in human tissues.

10.2.2a Western blots.

Fig.10.3 shows the distribution of 11ß-HSD by western analysis using the anti-11ß-$HSD2_{370-383}$ antibody (see Chapter 9) on 25000g pellet fractions (mitochondria and heavy microsomes) of a range of human tissues. Placenta was obtained fresh at delivery, liver and kidney were obtained fresh at surgery (although 48hrs post-mortem specimens showed similar findings) Salivary gland(lane 8) obtained at surgery showed a band at 40k, whilst post-mortem salivary gland specimens often show varying proportions of 40k and 31k bands. As shown in fig.10.3 lane 10, the 31k band may appear alone. The other tissues were from post-mortem at 48hrs from organs without major degradation visible macroscopically. There was no evidence of 11ß-HSD2 in liver, spleen, heart, adrenal or lung. A band at ≈70k was visible in liver. Further analysis revealed this was more abundant in liver homogenate than 25000g fraction and so has a different subcellular localisation to 11ß-HSD2.

10.2.2b Immunohistochemistry.

Immunohistochemistry on human placenta reveals dense immunostaining of trophoblast (fig.10.4) in the syncytiotrophoblast layer (but not decidua). Very dense immunostaining in adult human kidney(fig10.5) is seen localised to the distal nephron (distal convoluted tubule including juxtaglomerular loops, cortical and medullary collecting ducts). In both tissues the immunostaining was abolished by pre-absorption with the 11ß-HSD2(370-383) peptide. Neither tissue showed any immunostaining using pre-immune serum. The strength of immunostaining is not uniform across the cells around distal nephron tubules. Fig.10.6 shows cortical collecting tubules cut in section which clearly have abundant 11ß-HSD2 expression, however a minority of cells in the tubules, being ≈10%, have very little or no 11ß-HSD2 immunostaining.

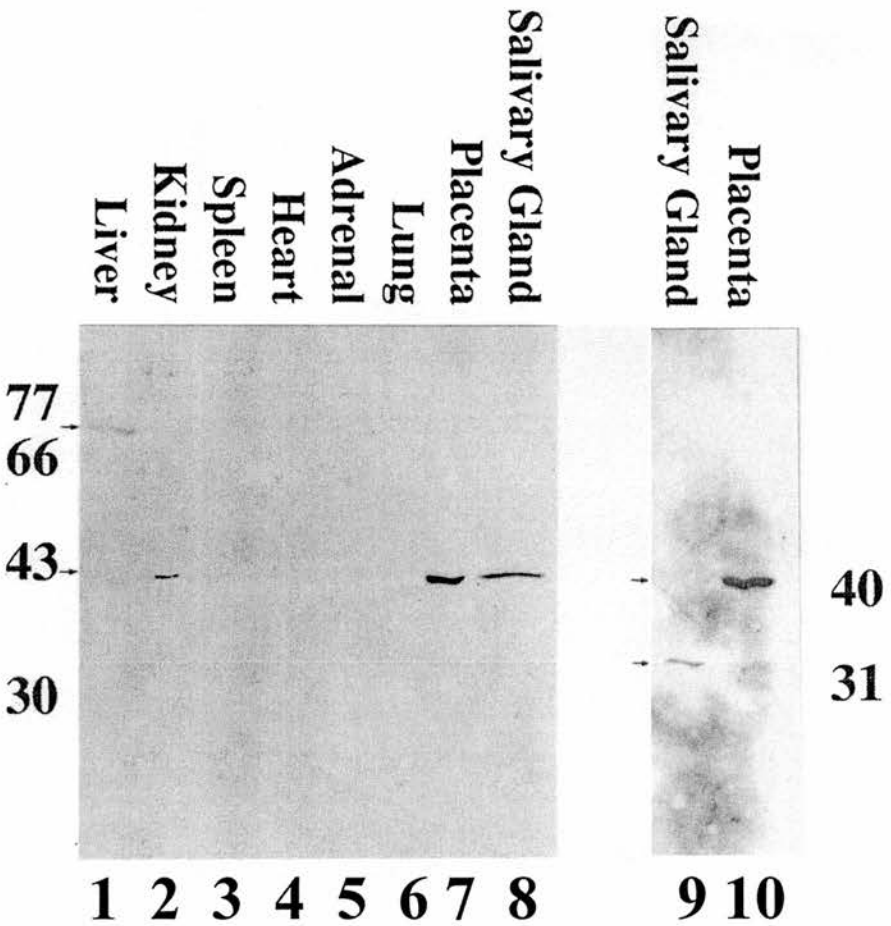


Fig. 10.3 Western blots using anti-11ß-HSD2 peptide$_{370-383}$ antiserum in human tissues. (*Left panel*) Fractions (25000g pellet) from a range of human tissues, lanes1-8 liver, kidney, spleen, heart, thyroid, lung, placenta and salivary gland. (*Right panel*) Crude homogenate salivary gland (lane9) and placental 25000g pellet (lane 10). Note salivary gland specimens show a ≈40k band (lane 8, 25000g pellet) or ≈31k band (lane 9, crude homogenate), the latter being a common finding in crude homogenates of post-mortem specimens of salivary gland tissue. (lane 8, ≈40k protein band) was obtained fresh from surgery whilst the sample in lane 9 (≈31k protein band) was obtained 48hours post-mortem.

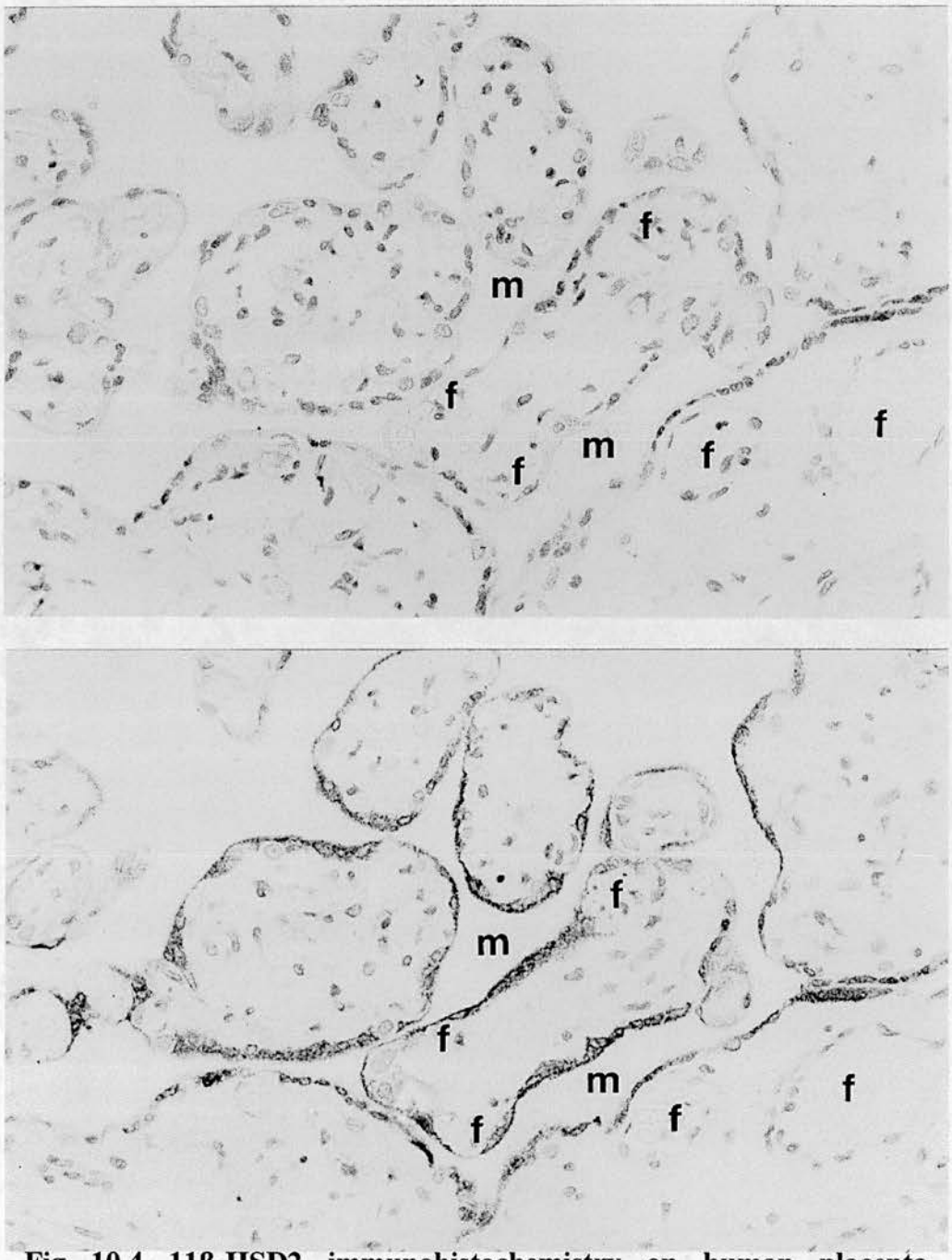


Fig 10.4 11ß-HSD2 immunohistochemistry on human placenta. Immunohistochemistry on human placenta with haematoxylin counterstaining (stains nuclei) showing high power view of placental villi cut cross-sectionally. Lower panel using antisera to human placental 11ß-HSD2 peptide 370-383. Upper panels, control with antisera pre-absorbed with the 11ß-HSD2 peptide 370-383. Fetal blood circulates in fetal capillaries (marked 'f ') within the villi, maternal blood occupies the intervillus space (marked 'm'), whilst abundant 11ß-HSD2 expression is localised in the syncytiotrophoblast layer which intervenes between fetal and maternal circulations and is seen as a dark band at the perimeter of the villi.

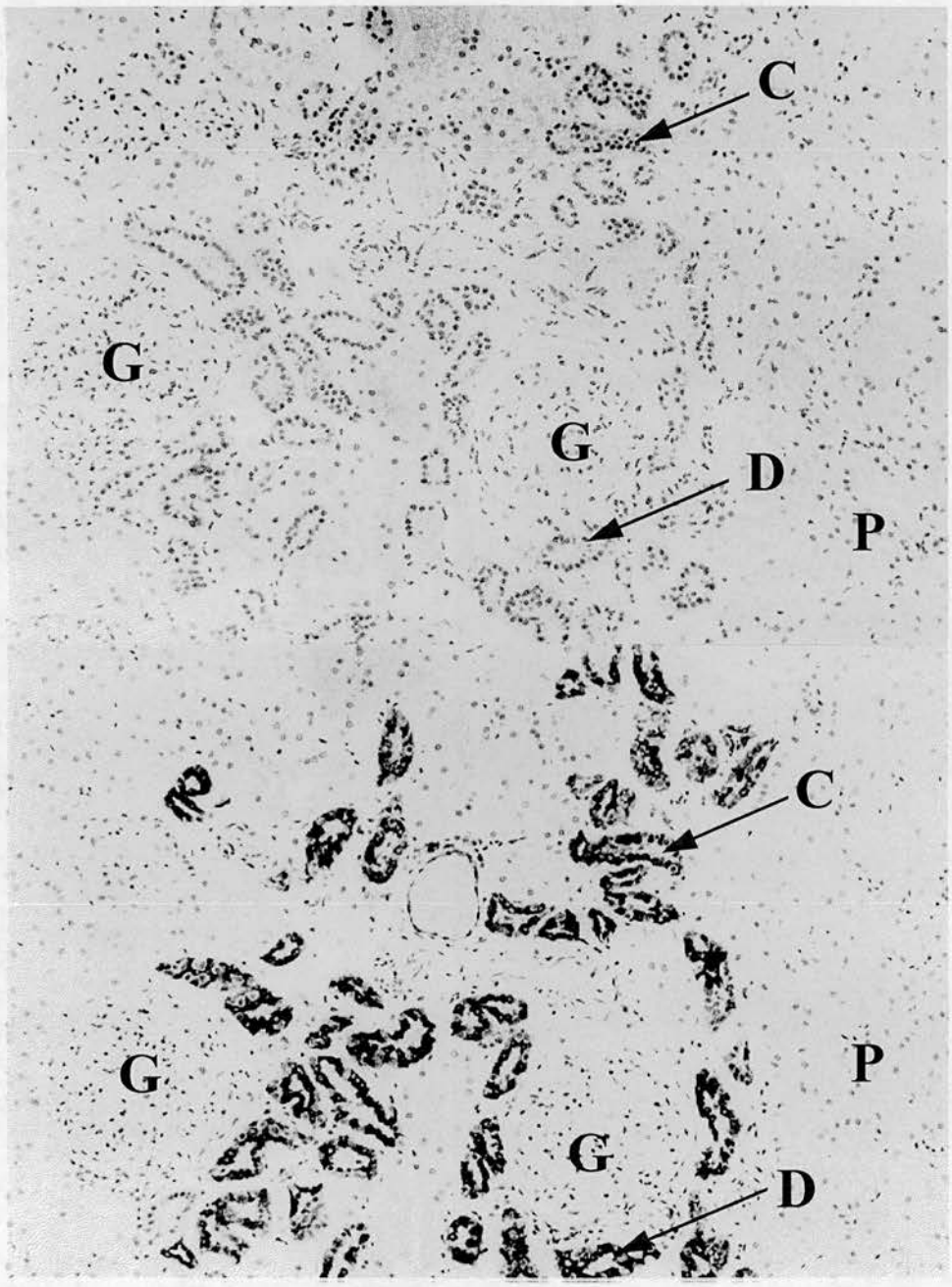


Fig 10.5 11ß-HSD2 immunohistochemistry on human kidney. Immunohistochemistry on human adult kidney cortex with haematoxylin counterstaining (stains nuclei) the section is shown at medium power. Lower panel using antisera to human placental 11ß-HSD2 peptide 370-383. Upper panel, control with antisera pre-absorbed with the 11ß-HSD2 peptide 370-383. The following are labelled: G, glomerulus; D, distal convoluted tubule; P, area with proximal tubules; C, cortical collecting duct. Clearly the 11ß-HSD2 protein is abundant in distal nephron.

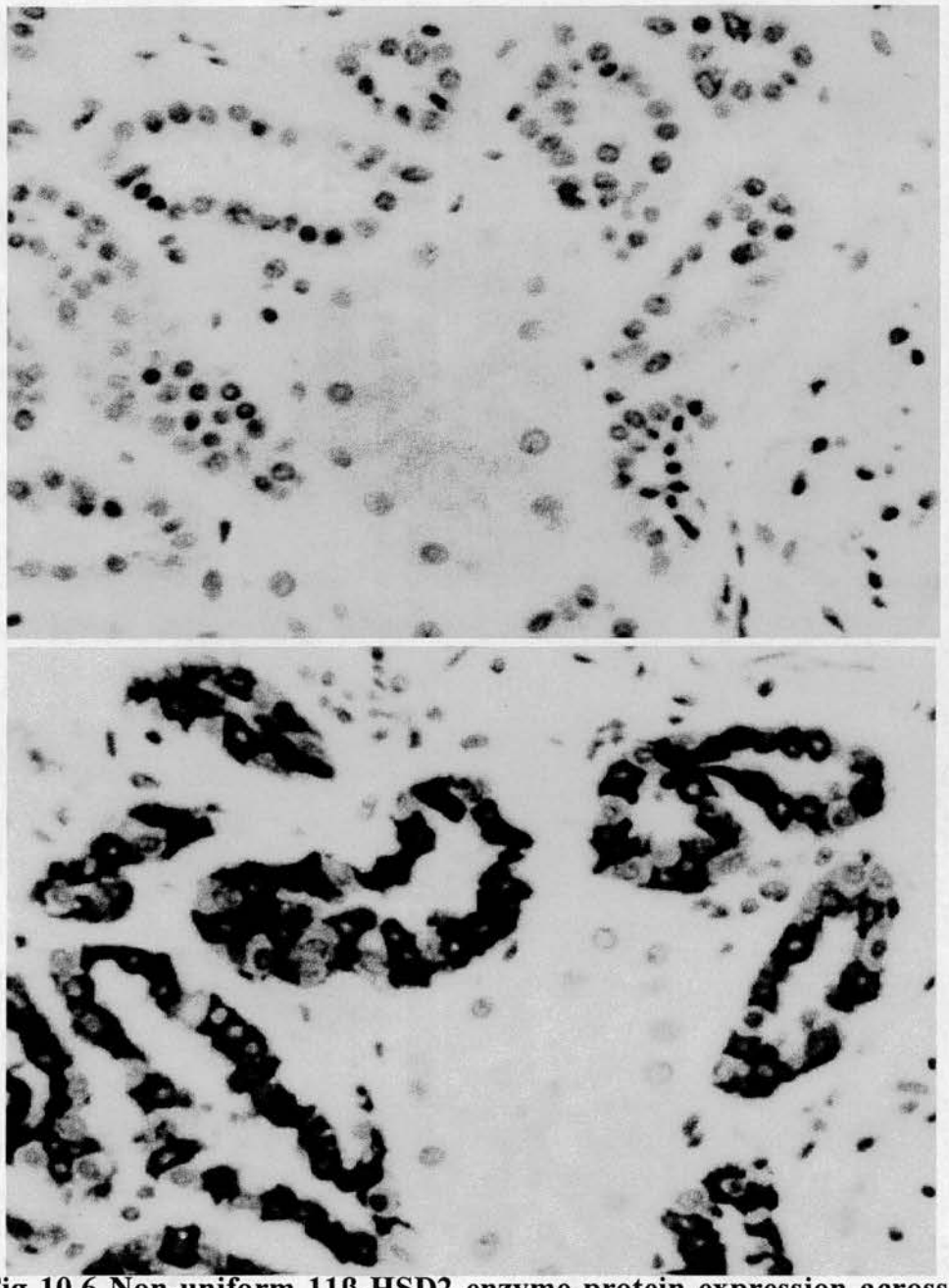


Fig 10.6 Non-uniform 11ß-HSD2 enzyme protein expression across epithelial cells of distal nephron. Immunohistochemistry on human adult kidney cortex [with haematoxylin counterstaining; (stains nuclei)] at high power showing the non-uniform 11ß-HSD2 expression around tubules of the distal nephron. See fig 10.5 for lower power view. Lower panel using antisera to human placental 11ß-HSD2 peptide 370-383. Upper panel, control with antisera pre-absorbed with the 11ß-HSD2 peptide 370-383. Clearly in a minority of distal nephron cells the 11ß-HSD2 enzyme is absent or is expressed at low levels compared to the majority of epithelial cells in such tubules.

10.3 Discussion.

11ß-HSD2 mRNA (1.9kb transcript) is expressed in placenta, aldosterone target tissues (kidney, parotid, colon and skin) and pancreas. Expression in pancreas was something of a surprise and is most likely to be in the exocrine gland. As MR are reported to be expressed in pancreas this raises the possibility that 11ß-HSD2 may be controlling MR specificity in this organ too possibly thereby regulating electrolyte aspects of pancreatic fluid composition..

11ß-HSD2 transcripts are expressed in fetal kidney (but not lung, liver or brain at 21-26weeks), suggesting an 11ß-HSD2 distribution resembling that in adult is established by this stage in human development. Two studies, using tissue slices at 10-20 weeks gestation[26] or adding NAD to tissue homogenates from 16-19weeks[67], have found substantial levels of 11ß-dehydrogenase activity in almost all human fetal tissues (including kidney, lung and brain). Fetal liver was found to have net 11ß-reductase in the first study and a NAD-dependent 11ß-dehydrogenase in the second. We find no 11ß-HSD2 hybridisation in fetal lung, liver or brain. As 11ß-HSD1 mRNA is reported absent, at least in the fetal lung and liver[67], it is possible a third 11ß-HSD isoform is expressed in fetus. However, as our fetal samples were from slightly later in gestation it is possible that 11ß-HSD2 expression, while present in early gestation, is switched off in many fetal tissues (kidney being a clear exception) at mid-gestation (specifically before 22-23weeks in lung, 22-26weeks in liver and 21-26weeks in brain). Indeed, this interpretation is now supported by our subsequent work[194] on mouse development (see Chapter 11, s.11.1). The mechanism of such developmental control is unknown but interestingly it seems a CpG island may be associated with 5' regulatory regions of the 11ß-HSD2 gene because:- (1) the CpG island extends to the very start of the 11ß-HSD2 clone, (2)from the 11ß-HSD2 transcript size on northern blots it appears the p11ß2 clone must be approximately full length (although we have not precisely mapped the start site) and (3) CpG islands are large (typically >1kb) and often include the most 5' exon as well as upstream sequences[111]. This is intriguing because CpG islands are often methylated in tissues where the gene is not expressed. Silencing of the expression of genes with 5' CpG islands may be associated with methylation of the CpG island. Whether regulatory influence of this kind occurs in the 11ß-HSD2 gene remains to be elucidated. Work on baboon pregnancy suggests that the 11ß-HSD dehydrogenase barrier to maternal glucocorticoids

only becomes firmly established after mid-gestation[76,113]; it may be therefore that prior to this fetal tissues may express 11ß-HSD2 and "protect themselves" from high maternal glucocorticoid levels. As the placenta takes over the protective role, fetal tissues adopt a much more 'adult' 11ß-HSD2 expression pattern and fetal adrenal activity may begin to dictate their glucocorticoid exposure.

The nature of the 4kb 11ß-HSD2-hybridising transcript in fetal kidney is unclear. Interestingly, flanking duplications involving the genes encoding human steroid metabolising enzymes are common (e.g. 17ß-HSD type1, the 3ß-HSD family, 5 - reductase type 1, 21-hydroxylase and 17ß-HSD type4), and duplicates are often transcribed, as separate transcripts or, in the case of 17ß-HSD4, as a large transcript traversing the apparent gene duplication.

In situ hybridisation and immunohistochemistry demonstrate abundant 11ß-HSD2 mRNA and protein in kidney in the location expected (distal nephron) for 11ß-HSD2 to confer aldosterone selectivity on MR. 11ß-HSD2 expression in distal convoluted tubule extends into loops participating in juxtaglomerular complexes. On closer inspection of the pattern of immunostaining in the distal nephron it is clear that a minority of the tubular epithelial cells have little or no 11ß-HSD2. This finding is fully reproducible. In such tubules it is known that there are principal cells, α-intercalated cells and ß-intercalated cells. The rarest of these are the α-intercalated cells which, based on rabbit and some rodent studies, seem to comprise ≈10%-20% of outer cortical collecting tubular epithelial cells[182,184,185]. These are involved much more with acid excretion/bicarbonate reabsorption. From the distribution it seems highly likely 11ß-HSD2 activity in human distal nephron is much higher in principal and ß-intercalated cell types than α-intercalated cells. This is in keeping with findings from cell sorting of immunodissected rabbit cortical collecting duct[11] where it was found that both principal cells and ß-intercalated cells expressed NAD dependent 11ß-HSD activity (no comment being made about α-intercalated cells).

In placenta 11ß-HSD2 expression was abundant in the syncytiotrophoblast lining the placental villi; thus being at the very interface between fetal tissue and maternal blood, which flows in the intervillus spaces. This is exactly the location which would allow 11ß-HSD2 the greatest influence over the passage of maternal glucocorticoid to the fetus.

Studies with western blots revealed 11ß-HSD protein in placenta and aldosterone target tissues (kidney and salivary gland) but not in the other tissues including adrenal and lung. 11ß-HSD assays on these tissues showed NAD-dependent 11ß-HSD activity only in the same tissues which were positive for 11ß-HSD2 on western blot. NADP-dependent 11ß-HSD activity was present especially in liver and to a lesser extent in lung and spleen indicating this was likely to be 11ß-HSD1. Thus human adrenal shows no evidence of 11ß-HSD2 on northern or western blot (and is also negative on *in situ* hybridisation). This is in striking contrast to the abundant 11ß-HSD2 expression in adrenal in the sheep [68,183]. The bands at ≈70k in liver and variably in kidney are of uncertain origin. The band in liver seems not to be 11ß-HSD2 related as it has a different subcellular distribution and there is no evidence for 11ß-HSD2 mRNA or enzyme activity(see Chapter 4) in liver. In kidney the origin of the weak ≈70k band is less clear. The finding of a large ≈4kb transcript in human fetal kidney(fig.10.1) hybridising to 11ß-HSD2 probes on northern blot is intriguing but currently there is no evidence to suggest a definite relationship between this larger transcript and the 70k protein in kidney. A similar large transcript hybridising to 11ß-HSD2 has also been recently reported in adult kidney in the mouse [145].

Chapter 11 : General Discussion.

11.1 Completion Of The Aims Of The Thesis.

The original aims of the thesis, which were the purification, cDNA cloning and raising of an antibody to the isoform of 11ß-HSD in placenta and distal nephron(now called 11ß-HSD2), have been completed and in addition a novel method of specifically identifying the human 11ß-HSD2 enzyme has been developed. This work has allowed detailed analysis of the 11ß-HSD2 enzyme protein structure and study of the recombinantly expressed 11ß-HSD2 protein and its enzyme activity. The cDNA and specific anti-human 11ß-HSD2 antibodies generated during this work have also allowed study of the tissue distribution of the expression of this key enzyme at both the mRNA and protein levels. 11ß-HSD2 expression is found in mineralocorticoid target tissues and placenta as was expected. The demonstration of 11ß-HSD2 mRNA in human pancreas was not anticipated. It has been very difficult to obtain specimens of normal human pancreas with optimal preservation. To date we have not yet been able to demonstrate convincingly the localisation of 11ß-HSD2 protein in human pancreas by immunohistochemistry nor has there been clear evidence of high 11ß-HSD2 activity on assay of the samples we have obtained. Currently it is unclear whether the 11ß-HSD2 enzyme protein has degraded too quickly to detect (proteolysis of course being a particular problem in pancreas),or whether the 11ß-HSD2 mRNA is largely untranslated in this tissue. In the rat, another group has reported immunohistochemical localisation of 11ß-HSD2 in the exocrine pancreas [193]. MR expression has also been reported in rat exocrine pancreas[12] and so these findings suggest the possibility that in the rat 11ß-HSD2 may be acting to protect MR in pancreas as well; possibly regulating the water and electrolyte composition of pancreatic fluid. It seems likely this is the case in humans too. However, this is not yet proven and species differences concerning 11ß-HSD2 expression have already been noted for the adrenal gland (11ß-HSD2 being reported absent from the human[102] and mouse[145] adrenal but present in sheep[68,183] and rat [192]) and so extrapolation between species may not always be straightforward. 11ß-HSD2 gene expression was also present in fetal kidney at 22-26wks and the 11ß-HSD2 expression in term placenta was shown to be in the syncytiotrophoblast, which is the fetal portion of placenta. Interestingly, there was no 11ß-HSD2 expression in some other human fetal tissues at 22-26wks (liver, lung and 'brain') as discussed in Chapter 10, these

findings, when compared with findings from enzyme assay of fetal tissues in previous literature[26][67], suggest that a silencing of 11ß-HSD2 gene expression occurs in several tissues during human development after mid-gestation. We have now carried out extensive *in situ* hybridisation studies during mouse development to determine the ontogeny of 11ß-HSD2 and find there is indeed a dramatic cessation of 11ß-HSD2 gene expression in many tissues between embryonic day 12.5 (E12.5) and E13.5 (19 days being full gestation in the mouse)[194]. Kidney is a notable exception with 11ß-HSD2 expression persisting to full-term and continuing post-natally into adulthood. These findings strongly support our hypothesis of a similar silencing of 11ß-HSD2 expression in the second half of human gestation in many fetal tissues with expression persisting in kidney.

11.2 Insights into 11ß-HSD2 Enzyme Structure and Function.

112.1 Requirement for Membrane Components for Stability.

Purification of 11ß-HSD2 to homogeneity proved to be a formidably difficult task, principally because the enzyme activity became labile on attempting solubilisation with all of the wide range of detergents screened. This suggests the active 11ß-HSD2 enzyme protein has an ordered association within the membranes in which it resides and that detergent disrupts this so leading to loss of stabilising components and increased exposure of the 11ß-HSD2 protein to inactivating processes. In particular, it seems that 11ß-HSD2 requires constituents of placental membrane fractions not adequately substituted by a wide range of detergents. As discussed in Chapter 7, 11ß-HSD2 has a highly positively charged structure and these charges are likely to cluster or align along faces of the protein so making associations with neighbouring negatively charged molecules likely. Thus stabilisation of 11ß-HSD2 by negatively charged moieties of phospholipids seems particularly likely and such stabilisation has been shown for the two related SCAD enzymes 3hydroxybutyrate dehydrogenase[95] and human 17ß-HSD1 [195]. 3hydroxybutyrate dehydrogenase requires negatively charged phospholipid for activity[95], whilst the crystal structure of human 17ß-HSD1 points to the positive charge clusters towards its C-terminus interacting with phospholipids so stabilising a domain which is otherwise very unstable and of such flexibility that it cannot be seen on crystal structure determined in the absence of membrane components[195]. Although there are compelling reasons to believe that

lipids are required to stabilise 11ß-HSD2 it is possible that other membrane proteins are involved(especially those with negatively charged regions). Indeed, it is clear from the purification of 11ß-HSD2 that several other membrane proteins of differing molecular weights extensively co-purified with 11ß-HSD2 while it remained highly active and when purification eventually substantially eliminated these co-purifying proteins 11ß-HSD2 had high lability or became inactive (see Fig.6.1).

11.2.2 11ß-HSD2 Substrate Binding and Reaction Mechanism.

The problems of lability and inactivation of highly purified 11ß-HSD2 necessitated a means of tracking 11ß-HSD2 when inactive. The novel affinity labelling technique developed allowed purification to homogeneity. The work with affinity chromatography and affinity labelling both give additional findings regarding the affinities of 11ß-HSD2 for its two substrates: dinucleotide cofactor (NAD) and glucocorticoids. This information allows conclusions to be drawn which may shed some light on the form of the enzyme's active site and kinetic mechanism. The striking specificity of the photoaffinity labelling of 11ß-HSD2 by corticosterone and cortisol, in the presence of NAD, demonstrates that 11ß-HSD2 exhibits an unusually high, receptor-like, affinity for these glucocorticoids, which may come close to that of GR or MR. This finding reflects the physiological role of 11ß-HSD2 in binding, and inactivating, glucocorticoids with high avidity before or in competition with GR and MR, so regulating glucocorticoid access to these receptors. 11ß-HSD2 is probably not the same as the 45k "low affinity" (K_d 100nM) dexamethasone binding proteins identified in studies on male rat liver[97] as it differs in localisation, hierarchical order of binding affinities and possibly Mg^{2+}-dependence of binding (11ß-HSD2 labelling occurs in EDTA containing buffers). However, 11ß-HSD2 does resemble another, incompletely characterised receptor/binding protein for glucocorticoids: the type 3 corticosteroid (corticosterone) receptor. Thus, the rank order of affinities determined in binding studies of steroids for the 'type 3 receptor sites'[22,98] is consistent with the order of potency of the steroids used to affinity label 11ß-HSD2(Fig. 5.1). 'Type 3 sites' often are regarded as cytosolic, which 11ß-HSD2 is not. However, the work originally defining them (in rat kidney) largely used supernatants from centrifugation at 30000g x 30min; which is very similar to the 25000g x 40min fractionation

we have used. Supernatant from such a spin will probably have 95% of protein from cytosol, but the remaining 5% will be rich in protein from 'light microsomes' and contain abundant 11ß-HSD2. Recent work, on isolated renal cortical collecting duct cells characterises a similar binding site in whole cells (where endogenous NAD may facilitate binding), which may be the renal 11ß-HSD2 isoform. The characteristics of placental 11ß-HSD2 are thus clearly similar to the 'type 3 sites' in these reports. In Chapter 8 this matter was addressed further by examining affinities of recombinant 11ß-HSD2. This reveals, amongst steroidal compounds, a similar rank order of potency as inhibitors of 11ß-HSD2 activity (Fig. 8.3) to the rank order of binding affinities described for 'type 3 sites'[22,98]. However at present it is not settled whether 'type 3 sites' and 11ß-HSD2 are the same entity.

The affinity chromatography data provide some clues to the reaction order and nature of the active site of placental 11ß-HSD2. It is likely that 11ß-HSD2 binding to N6-5'AMP-agarose was via an interaction at the cofactor binding site. 5'AMP is the 'half molecule' of NAD (nicotinamide comprising the remainder) and instances of N6-5'AMP affinity matrices interacting with the cofactor site of NAD-dependent dehydrogenases are well documented[84]. In Chapter 3 it was mentioned that the rank order of potency in which free cofactors elute bound human placental 11ß-HSD2 activity from N6-5'AMP agarose is NAD>NADH>5'AMP>>NADP (with 10-fold more 5'AMP required for elution than NAD). This indicates that the site at which 11ß-HSD2 binds the N6-5'AMP matrix behaves like a NAD cofactor site, as opposed to a 5'AMP allosteric site, as it has a clear preference for NAD(free cofactor). When cofactor is on the affinity matrix and not free, the rank order changes to N6-5'AMP(yield over 35%)>C8-NAD(yield 2%) > (N6-NAD = C8-5'AMP = NADP = nil). Clearly the attached spacer arms make a considerable difference, sterically hindering NAD from binding more strongly than 5'AMP (as occurs with free cofactors). This may be a limitation of the particular affinity matrix products used or simply be because the larger size of NAD makes its binding more sensitive to steric hindrance. A further possibility is that NAD may cause a conformational change on binding (as quite frequently occurs with dehydrogenases), whereas 5'AMP, filling only a half site, does not. Thus, steric interference by the new conformation prevents 11ß-HSD2 binding to N6-NAD, but permits limited binding to NAD when linked at C8 (which has the spacer attachment rotated >90^{o} relative to N6, both

coming from the 5'AMP half of the NAD molecule). 5'AMP, causing no such change, binds well when N6-linked, but not at all when C8-linked.

The 5'AMP affinity chromatography results suggest NAD can bind first to 11ß-HSD2 (explaining both binding and elution with NAD). The NAD dependence of glucocorticoid affinity labelling suggests glucocorticoid cannot gain full access to the steroid binding pocket unless NAD has bound first. Under a range of elution conditions affinity chromatography with dexamethasone agarose was unsuccessful. The finding that under similar conditions 11ß-HSD2 is bound well by N6-5'AMP agarose, but not at all by dexamethasone agarose (though dexamethasone is a substrate) may possibly also reflect a 'cofactor first' binding order. By analogy with other dehydrogenases (e.g. lactate dehydrogenase[175]), these findings collectively suggest a compulsory ordered ternary complex mechanism may operate for 11ß-HSD2, with NAD binding first. Further studies of these matters will assist in mapping the active site and in design of drugs specifically to inhibit, or possibly constitutively activate, 11ß-HSD2.

11.3 11ß-HSD2 and Control of Blood Pressure.

It was clear from an early stage (Chapters 3 and 4, [60]) that the properties of the 11ß-HSD activity in human placenta suggested it was the same isoform as that predicted and later characterised in distal nephron[64,65]. Comparison of 11ß-HSD activity in placenta and kidney showed the major properties were the same [180], with minor differences again leading us to the conclusion the same isoform (11ß-HSD2), was present in both tissues. The human placental 11ß-HSD2 cDNA and the antiserum raised to human placental peptide sequence both detected 11ß-HSD2 in the expected site in kidney, confirming sequence similarity sufficient to result in high cross-reactivity. The sequence published for 11ß-HSD2 from human kidney[70] was not identical to the human placental 11ß-HSD2 cDNA described in this thesis. The renal cDNA has 2 deletions in the 3'UTR (bases 1270 and 1495: Fig.7.2) and a base substitution at position 442 resulting in a change in the predicted amino acid sequence, $148^{Val\ \ Leu}$. At the points of difference, the sequence reported above has been confirmed in cDNA we have sequenced derived from a second placenta, and the Val148 residue is clearly present in human placental 11ß-HSD2 as it is the first amino acid of the D peptide

sequence derived from purified 11ß-HSD2 tryptic digests(Chapter 6: Table.6.1). Moreover, in other work we have partially sequenced 11ß-HSD2 from the genomic DNA of over 20 individuals and always found the sequence is as given in this thesis, in particular amino acid 148 always being valine. This makes true microheterogeneity at this locus seem very unlikely and if the locus is polymorphic then we have found the prevalence of the 148^{Val} allele to be 100% in the individuals tested (all Caucasian or Hispanic origin). There is also the possibility that the variations are due to sequencing errors in the renal clone sequence. A human genomic 11ß-HSD2 sequence has been published by Agarwal *et. al.*.[146], but unfortunately the Genbank sequence files and the associated paper disagree with the paper having 148^{Val} while the Genbank sequence file has 148^{Phe}. The publication also identifies a different major start site for 11ß-HSD2 transcription in kidney and placenta. This is in keeping with the fact that the renal cDNA lacks the first 25 bases of the placental cDNA given above in Fig.7.2. However the putative placental start site described cannot be the only one as the human placental 11ß-HSD2 cDNA described in Chapter 7 begins upstream of it. Two groups have now determined the chromosomal locus of 11ß-HSD2 as 16q22[146,147].

From the above it can be seen that it is most likely that the 11ß-HSD2 enzyme activity in placenta and kidney are derived from the same gene, driven by different promoters, but with the same coding region. It was clearly of great interest to determine whether this gene was mutated in the human hypertensive syndrome SAME. Two groups reported, approximately simultaneously, describing inactivating mutations in SAME patients[148,199]. Other subsequent reports have found further 11ß-HSD2 mutations in SAME patients [196-198]. Thus it is established, beyond doubt, that 11ß-HSD2 is the tissue specific protector of the mineralocorticoid receptor and that normal activity of this enzyme is required to maintain normal systemic arterial blood pressure and electrolyte balance. 11ß-HSD2 thus becomes one of the few genes proven to be the cause of human hypertensive syndromes and now stands alongside the amiloride-sensitive sodium channel (the cause of hypertension in Liddle's syndrome[200,201]) and aldosterone-synthase (a natural fusion mutation of which is the cause of hypertension in glucocorticoid remediable hyperaldosteronism [202,203]) as genes which may also contribute to the causation of essential hypertension. Thus, it is expected there will be

studies looking for mutations in 11ß-HSD2 not only to explain the dramatic loss of 11ß-HSD2 enzyme activity that occurs in SAME but also where there are more subtle variations in 11ß-HSD activity such as those indicated by the altered urinary glucocorticoid metabolites described in (i)subsets of patients with essential hypertension [72] or (ii)polycystic ovary disease [66]. Moreover, as mentioned in Chapter 1 (section 1.3) deficient placental 11ß-HSD2 activity has been implicated in the link between the in utero environment and the programming of low birth weight and subsequent hypertension in humans [77]. The work of this thesis has produced important tools (cDNA, specific antibody and 11ß-HSD2 sequence) for further study of this important enzyme and these may help shed further light on the fundamental processes underlying the control of the level of blood pressure in humans and how these are distorted in human hypertensive states including essential hypertension.

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Human Placental 11β-Hydroxysteroid Dehydrogenase: Evidence for and Partial Purification of a Distinct NAD-Dependent Isoform*

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ABSTRACT

Excess glucocorticoids impair fetal growth and cause teratogenesis. Placental 11β-hydroxysteroid dehydrogenase (11βHSD) catalyzes the inactivation of cortisol to cortisone, preventing the high maternal cortisol levels from reaching the fetal circulation and thus preserving the low cortisol fetal environment. In previous work, an NADP-dependent isoform of 11βHSD has been purified from rat liver, a cDNA isolated, and the human homolog cloned. However, much evidence suggests tissue-specific 11βHSD activities that cannot be explained by the liver-type isoform. Therefore, we have partially purified human placental 11βHSD and compared it to the enzyme in rat liver. Human placental subcellular fractions exhibited NAD-dependent 11βHSD activity, but showed little activity with NADP. The enzyme had a pH optimum of 7–8.5 (peak, 7.7), was only sparingly soluble in detergents (solubility with Triton X-100 was very poor), and exhibited little latency or change in pH profile in detergent solution. By contrast, rat liver 11βHSD was exclusively NADP dependent and was easily solubilized by a wide range of detergents (including Triton X-100), revealing substantial latency and altered pH profile [optimum of 10, becoming 7–10 (peak, 9.5) in detergent]. These data do not merely reflect species differences, as rat placental 11βHSD was similiar to the human placental isoform. AMP affinity chromatography, which was completely without affinity for rat liver 11βHSD, achieved a 1000-fold purification of human placental 11βHSD. This had K_m values for corticosterone (mean ± SE, 14 ± 1 nM) and cortisol (~55 nM) that were over 100 times lower than that for liver 11βHSD. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis allowed identification of a band (apparent mol wt, 40,000) that correlated consistently with human placental 11βHSD activity (contrasting with a mol wt of 34,000 for rat liver 11βHSD). Thus, the NAD-dependent human placental 11βHSD is distinct from the previously characterized rat liver isoform and may be the product of a separate gene. (*Endocrinology* **132:** 2614–2621, 1993)

11β-HYDROXYSTEROID dehydrogenase (11βHSD) plays a crucial role in corticosteroid physiology by regulating glucocorticoid access to both glucocorticoid and mineralocorticoid receptors. It achieves this by catalyzing the rapid conversion of active physiological glucocorticoids (cortisol and corticosterone) into inactive 11-dehydro products (cortisone and 11-dehydrocorticosterone) (1, 2). Indeed, in the classic aldosterone target tissue, renal distal convoluted tubule (DCT), it is 11βHSD that ensures mineralocorticoid receptors, which are nonselective *in vitro* (binding aldosterone and physiological glucocorticoids with equal affinity), achieve aldosterone selectivity *in vivo* in the face of 100- to 1000-fold excess of circulating glucocorticoids (1, 2). 11βHSD from rat liver has been purified (3), and a cDNA isolated (4) and recombinantly expressed (4, 5), allowing this NADP-dependent enzyme to be characterized. However, within several tissues, particularly the distal nephron, there are striking discrepancies between the presence of 11βHSD enzyme activity and the absence of the liver-type protein (by immunohistochemistry) (1, 6) or liver-type (1.7-kilobase) mRNA (7, 8). This and other evidence suggesting the presence of variant tissue-specific forms of 11βHSD (8–12) have led to speculation about a second 11βHSD enzyme (11βHSD2) (13, 14). Recent work on renal DCT has particularly supported this view, with the finding of an apparent NAD-dependent 11βHSD isoform detectable by histochemical activity staining (15, 16) and direct assay (14).

Placental 11βHSD is of particular importance, as it regulates the transfer of maternal glucocorticoids to the fetus (17–19). Excess glucocorticoids may cause deleterious effects on the fetus, impairing fetal growth and causing teratogenesis, and, moreover, may predispose the individual to hypertension in adulthood (17). Despite its physiological importance, placental 11βHSD has been relatively poorly characterized and has not been purified. Furthermore, there is a long-standing controversy as to its cofactor dependence (19–23), with some studies finding NADP preference [in homogenates (21, 22) and microsomes (19)], some equal preference [in microsomes (20)], and others NAD preference [in microsomes (23)]. Therefore, we have partially purified and characterized 11βHSD from human placenta. We present evidence of an abundant NAD-dependent 11βHSD activity in human placenta that represents an isoform distinct from that previously

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characterized from rat liver microsomes (3, 24). To determine if this is solely due to differences between species, we have tried, where possible, to present parallel findings in the rat placenta.

Materials and Methods

[1,2,6,7-^{3}H]Corticosterone and [1,2,6,7-^{3}H]cortisol (SA, 78 and 73 Ci/mmol, respectively) were obtained from Amersham International (Aylesbury, Buckinghamshire, United Kingdom). NAD, NADP, NADH, NADPH, AMP, AMP-agarose, dithiothreitol, EDTA, Triton X-100, 3-[(3-cholamidopropyl)dimethylammonio]1-propanesulphonate (CHAPS), and porcine heart mitochondrial malate dehydrogenase were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Glycerol and Electran protein electrophoresis mol wt standards were purchased from BDH Laborotory Supplies (Poole, Dorset United Kingdom). Coomassie blue dye concentrate and standardized BSA were purchased from Bio-Rad (Hemel Hempstead, United Kingdom). HPLC grade methanol and water were purchased from Rathburn Chemicals (Walkerburn, Scotland, United Kingdom), and Quickszint 302 HPLC scintillant from Zinsser Analytic (Maidenhead, Berks, United Kingdom).

Buffers

Buffer systems used were as follows: buffer A, 20% glycerol, 5 mM potassium phosphate (pH 7.0), 1 mM EDTA, and 1 mM dithiothreitol; buffer B, 10% glycerol, 300 mM sodium chloride, 4 mM CHAPS, 1 mM EDTA, and 0.02 M Tris-HCl (pH 7.7; buffer B2, sodium chloride omitted); and buffer C, 10% glycerol, 300 mM sodium chloride, 1 mM EDTA, and 0.02 M Tris-HCl (pH 7.7). Where pH was varied, buffers included 10% glycerol, 1 mM EDTA, 300 mM sodium chloride, and 0.1 M buffer: pH 5–6, potassium acetate; pH 6.5–7.5, potassium phosphate; pH 7.5–9, Tris-HCl; pH 9.5–10, potassium carbonate; and pH 10.5–11, glycine-NaOH. Buffers were pH adjusted at the temperature of use.

Tissues and tissue processing

Rat livers and placentae were obtained from adult male and female (20–21 days pregnant) Wistar rats, respectively. Fasted animals were killed by decapitation. Human term placentae (400–600 g) were obtained at normal vaginal delivery. All tissues were rapidly placed on ice. Tissue processing was begun within 2 h of placing on ice. Loose membranes and umbilical cord were trimmed from placentae. Adherent membranes and large vessels were also trimmed off human placentae. All tissues were minced with scissors and washed in ice-cold 0.9% saline, blotted dry, suspended in approximately 3 times their weight of buffer A, and homogenized with a Ystral homogenizer (Scientific Instrument Centre, Liverpool, United Kingdom) (rat tissue) or commercial blender (human placental tissue). Human placental homogenate was filtered through two layers of muslin. For rat placentae, to aid fractionation, the homogenate was diluted 2-fold (with buffer A), rather than being filtered, so as to minimize losses from the small tissue quantities. Homogenates were subjected to subcellular fractionation without delay by a differential centrifugation protocol (similiar to that described in Ref. 23): 1) 750 × *g* for 10 min, 2) 25,000 × *g* for 40 min, and 3) 110,000 × *g* for 60 min. The supernatant from each spin was subjected to the next centrifugation, finally leaving cytosolic supernatant (discarded). The resulting pelleted fractions of human placental or rat liver tissue were frozen and kept at −80 C if not used immediately. Rat placental fractions were not frozen, but placed on ice and used rapidly.

Assays of 11βHSD activity

C-11 dehydrogenase activity was determined by measuring the rate of conversion of 1.12×10^{-8} M [^{3}H]steroid substrate (corticosterone or cortisol) to product (11-dehydrocorticosterone or cortisone, respectively) in the presence of 400 μM NAD or NADP (unless otherwise stated) and calculating the percent conversion during the assay. The 250-μl assay consisted of 10 μl containing tritiated steroid, 50 μl containing cofactor, and 190 μl enzyme in 0.1 M potassium phosphate (pH 7.5) and 300 mM sodium chloride buffer (buffer only in blank controls). Reactions were incubated at 37 C for 10 min and terminated by adding 2 ml ice-cold ethyl acetate and mixing. The organic layer was separated and evaporated, and the steroid was resuspended in 0.6 ml 50% methanol-50% water. Part (0.2 ml) of this was injected into a Berthold HPLC system (Berthold Instruments Ltd., St. Albans, Herts, United Kingdom) fitted with a Waters Associates μBondpack C18 column (Waters, Milford, MA), which was eluted with 50% methanol-50% water (vol/vol; at 1.8 ml/min for corticosterone substrate reactions). Eluted steroids were monitored by a UV absorbance detector and a Berthold LP506 C1 scintillation counter to detect tritiated steroids. The latter was flushed with Quickszint 302 scintillant (at twice the column elution rate), and output was calibrated against known authentic steroid standards. The percent conversion of steroid substrate to product was calculated as an index of enzyme activity. Cofactor concentrations and buffer pH were varied in experiments specifically examining these effects. The assay pH was verified by measuring the pH of a parallel mixture containing unlabeled steroid.

Protein was estimated by the method of Bradford (25), using Bio-Rad protein dye (Bio-Rad) and calibration against standards of BSA. Preliminary work identified optimum assay conditions for each tissue, so that the amount of protein added 1) was in the linear region of the curve of protein concentration *vs.* percentage substrate converted with 400 μM of the more active cofactor (NAD or NADP), and 2) resulted in a percent conversion of 10–40% in 10 min. All experiments had blank (no protein) assays run in parallel to establish the background level of steroid product. 11β-Reductase activity was measured using the same reaction conditions, except 400 μM NADH or NADPH and [^{3}H]11-dehydrocorticosterone were used as substrates. The latter was prepared from [^{3}H]corticosterone by a variation of the method of Lakshmi and Monder (3), using human placental enzyme with NAD at pH 7.5.

Kinetic parameters were calculated from the initial velocity determinations obtained from experiments performed with a wide range of substrate concentrations. Inhibition by 0.06–2 mM CHAPS was also studied. Enzyme concentrations giving less than 30% conversion at 10 min were used.

Detergent solubilization of 11βHSD activity

All steps were carried out at 0–2 C. Tissue fractions, resuspended in buffer C to 6 mg protein/ml, were mixed with an equal volume of solubilization buffer (buffer C with detergent at twice the final concentration). After 30 min, the mixture was centrifuged at 110,000 × *g* for 1 h. Supernatant containing soluble enzyme was carefully removed. It was found that 4 mM CHAPS was optimal for solubilization of human placental 11βHSD, and 0.06% Triton X-100 (final detergent/protein ratio of 0.2) was optimal for solubilizing microsomal rat liver 11βHSD [in agreement with Lakshmi and Monder (24)].

Affinity chromatography

One or 5 ml AMP-agarose equilibrated with buffer B was used. The CHAPS-solubilized tissue fraction containing 11βHSD activity was loaded onto the column, which was then washed with buffer B. 11βHSD was eluted with nucleotide cofactor in buffer B, as indicated in the text. Fractions (1.5 ml) from the column were collected, placed on ice, and rapidly assayed for 11βHSD enzyme activity. In some experiments, a 0- to 3000-μM gradient of NAD, AMP, or NADP in buffer B2 was applied to the column across a total of 10 column vol (50 ml) After completion of the gradient, the column was washed with buffer B, and any remaining 11βHSD was eluted with buffer B containing 3 mM NAD. This protocol was designed to determine thresholds of elution with different cofactors, allowing their affinities for the active site of the enzyme to be compared (26, 27).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (28) with 12.5% resolving gels (3.3% cross-linker). Fractions with low protein

levels were concentrated by acetone precipitation. Gels were silver stained by the method of Wray *et al.* (29), with variations of the NH_4^+/Na^+ ratio to minimize the chances of protein being undetected.

Removal of NAD from affinity-purified 11βHSD

NAD was removed from partially purified 11βHSD enzyme (eluted as described above) by three successive filtration/dilution cycles in a Centricon-10 concentrator (Amicon, Lexington, MA). After filtration by centrifugation at 4600 × *g* for 90 min, samples were diluted in buffer B, and the cycle was repeated. Removal of NAD was verified by measuring A_{280}. During this process, samples were assayed for 11βHSD activity to determine dependence on added cofactor.

Statistics

Results are shown as the mean ± SE. Series of paired samples were compared by one-way analysis of variance of their differences. Kinetic parameters were estimated by Lineweaver-Burke and Eadie-Hofstee analyses facilitated by computer-generated linear regression.

Results

For all three tissues, [^{3}H]corticosterone and [^{3}H]cortisol produced qualitatively similiar results, but [^{3}H]corticosterone was clearly a better substrate for 11βHSD than [^{3}H]cortisol (as illustrated for human placenta in Fig. 1A). Moreover, separation of substrate and product on HPLC was better in assays with [^{3}H]corticosterone. Thus, we have mostly used corticosterone in the studies presented here.

We concentrated on 11β-dehydrogenase in these studies, because 11β-reductase in the human placental tissue studied (full term placentae) was only a very minor activity (<5% of 11β-dehydrogenase at pH 7.5). Rat placental reductase was even less abundant. Moreover, the activity of placental 11βHSD reductase was unstable and underwent a progressive decline at 37 C. This attenuation of activity was detectable even at 10 min.

Preliminary subcellular localization of placental 11βHSD

Human placental 11βHSD was most abundant in the 25,000 × *g* pellet, which contained over two thirds of the total 11βHSD activity, 5-fold higher than the level in the microsomal 110,000 × *g* pellet. Moreover, the 25,000 × *g* pellet had as high a specific activity as the 110,000 × *g* microsomal pellet, and its activity was stable and more rapidly prepared. Differential centrifugation of the resuspended 25,000 × *g* pellet (which contains mitochondria and heavy microsomes) did not produce any useful refinement. Accordingly, this fraction was used for further studies on human placenta. The same fraction was used for rat placenta. Rat liver microsomes (110,000 × *g* pellet) were used, as these were the source of the purified enzyme (3) for which the encoding gene has been cloned (30, 31). Fractions selected for further study were assayed at protein concentrations of 2–5 μg/ml for human placenta, 20–50 μg/ml for rat liver, and 100–500 μg/ml for rat placenta; these gave percent conversions in 11βHSD assays in the desired range, as defined in *Materials and Methods*. Comparing the three tissues, rat placental 11βHSD was found to be the least abundant and most labile and was the only activity markedly attenuated by freezing and thawing. Accordingly, rat placental fractions were freshly prepared and used rapidly.

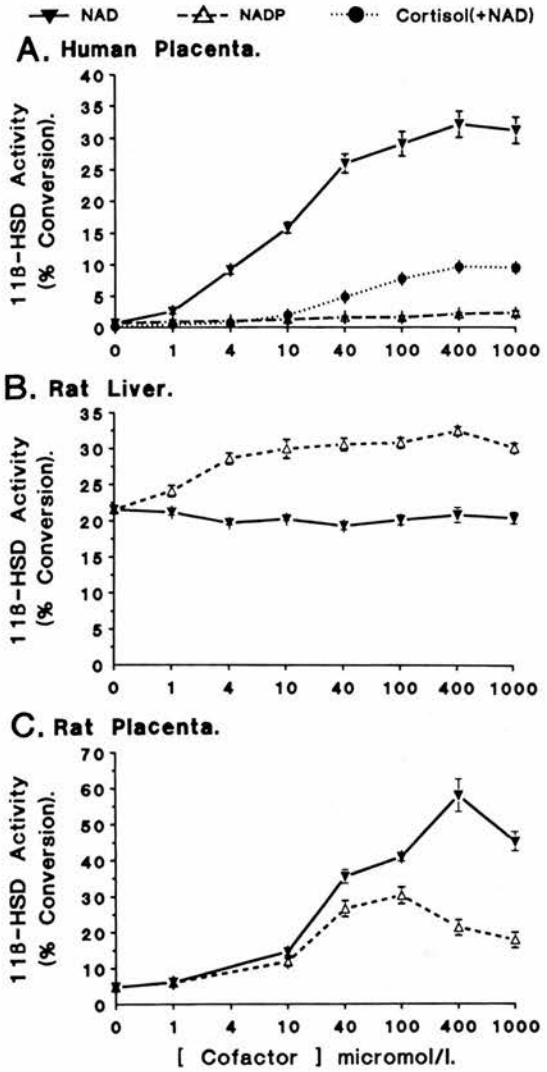

FIG. 1. Cofactor dependence of 11βHSD activity. 11βHSD activity (mean ± SE) in human placenta (A), rat liver (B), and rat placenta (C) with corticosterone as substrate in the presence of 0–1000 μM cofactor (NAD or NADP). Assays used placental 25,000 × *g* pellets and liver microsomes at protein concentrations of: human placenta, 2 μg/ml; rat liver, 50 μg/ml; and rat placenta, 500 μg/ml. Also shown is human placental 11βHSD activity with cortisol and NAD (with cortisol and NADP conversion remains <2%). Rat placenta, n = 4; otherwise, n = 5.

All further studies compared 11βHSD in the 25,000 × *g* pellet of placental tissues with rat liver microsomal 11βHSD.

11βHSD cofactor dependence

To define and compare the cofactor preferences of 11βHSD in the three tissues, the activity of 11βHSD was determined when the concentration of NAD or NADP varied across the range 0–1000 μM (Fig. 1). Both human and rat placental 11βHSD preferred NAD ($P < 0.001$ for both tissues, by analysis of variance), while the rat liver enzyme preferred NADP ($P < 0.001$). Activities with 100–1000 μM cofactor

were comparable in the three tissues (30–45% conversion), whereas with no cofactor, the activity was 22% conversion for rat liver and only 2% and 5% for human and rat placenta, respectively. Although there appeared to be no NAD-dependent 11βHSD activity in liver microsomes, the high activity without the addition of exogenous cofactor [which has been noted previously in liver (20)] made this difficult to assess. Minor NADP-associated 11βHSD activity was seen in human and rat placental fractions. Eadie-Hofstee analysis (not shown) of the data for human placental 11βHSD show rectilinear plots with NAD (apparent K_m for NAD, 13 μM;, maximum velocity, 180 pmol/mg protein·min), but curved plots with NADP, suggesting the possibility that two distinct enzymes (with low and high K_m values for NADP) contribute to the minor NADP-associated activity.

pH dependence of 11βHSD activity

The variation in 11βHSD activity in the three tissues over the pH range 5–11 is shown in Fig. 2. The similiarity between human and rat placental 11βHSD activities with either NAD or NADP is striking, as is their difference from 11βHSD activity in rat liver microsomes. Placental 11βHSD has a broad pH optimum of 7–8.5 with NAD (peak, 7.5–8 in human placenta). With NADP as cofactor, the optimum is more alkaline (pH 8–9). Above pH 9, placental 11βHSD activity falls off sharply (especially NAD-associated activity). In contrast, pH 9–10 is the optimum for rat liver 11βHSD. The activity of liver 11βHSD is largely or wholly independent of added NAD (Fig. 1B). The liver 11βHSD activity with added NAD may be due to a relatively high level of endogenous cofactor (32). Reductase activities for all three tissues were optimal at more acidic pH: pH 5 for rat liver 11β-reductase with NADPH and pH 5–6 for human placental reductase (data not shown). Solubilized 11βHSD pH profiles are discussed below.

Detergent solubilization

Detergents were used to solubilize 11βHSD, which was membrane associated in all three tissues. CHAPS (4 mM) was found to be optimal for solubilizing human placental 11βHSD. This is a lower concentration than that used in other studies on 11βHSD (24, 33). Triton X-100 has been shown to be a good detergent for solubilization of rat liver (24) and renal (33) 11βHSD. Triton X-100 (0.06%) was optimal for solubilizing rat liver microsomal 11βHSD activity. This corresponds to a detergent/protein ratio of 0.2, the optimum found by Lakshmi and Monder (24).

To compare detergent solubilities, all three tissues were treated with 1) 0.06% Triton X-100, 2) 4 mM CHAPS, or 3) buffer only. For each detergent, the proportion of total protein solubilized was similiar for all three tissues (~65% with Triton X-100 and 45% with CHAPS). 11βHSD assays were performed on samples taken 1) before centrifugation and from the detergent-solubilized fraction (110,000 × *g* supernatant) at 2) 1 h and 3) 24 h after centrifugation. The results for samples 1 and 2 are shown in Fig. 3. Solubilization of rat liver 11βHSD was very efficient compared to that of placental

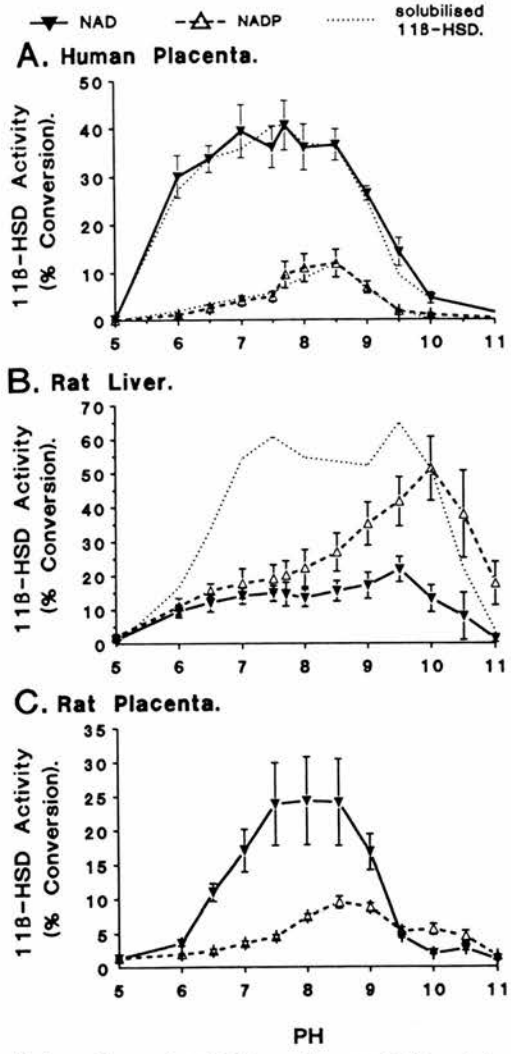

FIG. 2. pH dependence of 11βHSD activity. 11βHSD activity (mean ± SE) in human placenta (A), rat liver (B), and rat placenta (C) with corticosterone and 400 μM NAD or NADP across pH range 5–11. Assays used placental 25,000 × *g* pellets and liver microsomes at protein concentrations of: human placenta, 2 μg/ml; rat liver, 20 μg/ml; and rat placenta, 100 μg/ml. The *dotted line* shows the pH profile of solubilized enzyme (with 0.06% Triton X-100 or 4 mM CHAPS for rat liver and 4 mM CHAPS for human placenta). These are superimposed (to allow comparison of shape) to intersect the corresponding graph without detergent at its pH optimum (human placenta: NAD, pH 7.7; NADP, pH 8.5; rat liver: NADP, pH 10). Rat placenta, n = 4; human placenta and rat liver, n = 5.

11βHSD. The addition of either Triton X-100 or CHAPS increased rat liver 11βHSD activity (before centrifugation), demonstrating considerable latency. After centrifugation, a large proportion of the activity was soluble (between 90–100% of that before detergent, compared to <2% when no detergent was added).

In contrast, human placental 11βHSD showed minimal latency and was sparingly soluble (9%) in CHAPS, whereas the use of Triton X-100 did not significantly increase solubilized 11βHSD activity (1 ± 0.08% compared to 0.84 ± 0.02% without detergent). Rat placental 11βHSD behaved similiarly to human placental 11βHSD (compare Fig. 3, A

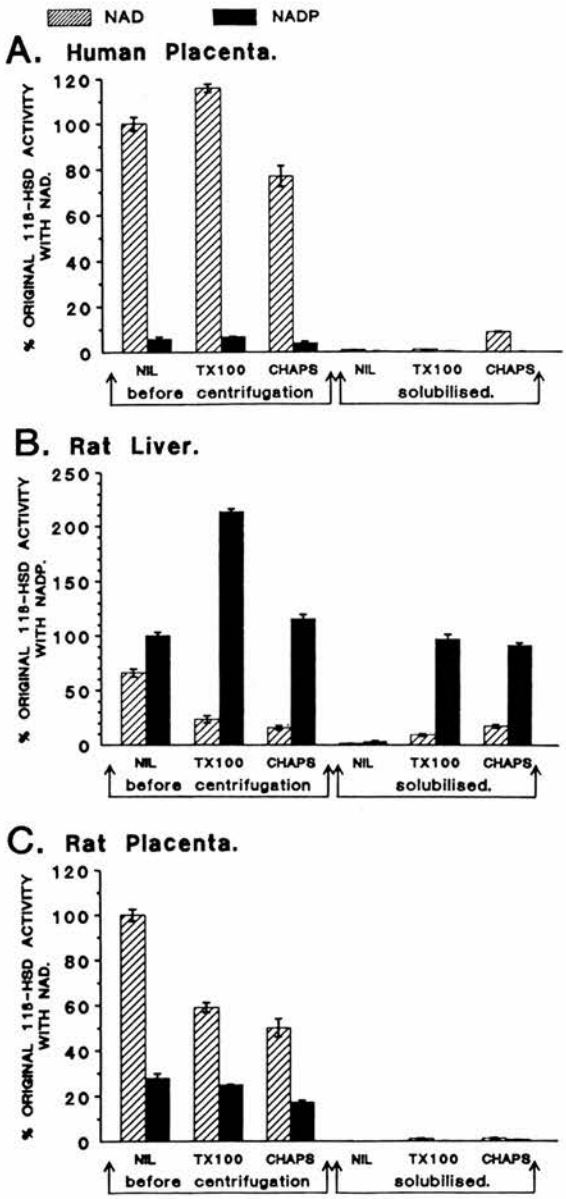

FIG. 3. Behavior in detergent of 11βHSD activity (mean ± SE) in human placenta (A), rat liver (B), or rat placenta (C) with corticosterone and 400 μM NAD or NADP. Tissue fractions (placental 25,000 × *g* pellets and liver microsomes) were each suspended at 3 mg/ml in buffer C only (NIL), buffer C and 0.06% Triton X-100 (TX100), or buffer C and 4 mM CHAPS and assayed first before centrifugation and then in supernatant after centrifugation at 110,000 × *g* for 60 min. Results are expressed as a percentage of the activity with preferred cofactor, without detergent and before centrifugation, with protein concentrations as follows: human placental, 2 μg/ml; rat liver, 20 μg/ml; and rat placenta, 300 μg/ml. Rat placenta, n = 4; otherwise, n = 10.

with C); however, analysis of rat placental 11βHSD was hindered by the lability of the enzyme. The soluble 11βHSD activities in both human placenta and rat liver extracts were stable, with more than 80% remaining after 24 h at 0–2 C. Solubilization changed the pH profile of rat liver 11βHSD, whereas very little change occurred when the human placental enzyme was solubilized. The altered liver 11βHSD pH profile remained very different from those of the placental enzymes in the pH 8.5–10 range (Fig. 2).

Thus, using the same detergent and protein concentrations, the liver and placental 11βHSD enzyme activities behaved very differently. Although human placental 11βHSD was difficult to solubilize, with reextraction of the initially insoluble 110,000 × *g* pellet, approximately 20% of the enzyme activity (from the 25,000 × *g* pellet) could be solubilized in a stable active form with 4 mM CHAPS, allowing further analysis.

Affinity chromatography

Human placental and rat liver CHAPS-solubilized extracts (containing solubilized 11βHSD) were subjected to AMP affinity chromatography. Sequential elution was performed with 1 mM NAD, NADP, and AMP (for human placenta; Fig. 4A), with NADPH following this for rat liver. A large proportion of the human placental 11βHSD activity eluted specifically with NAD (Fig. 4A). By contrast, liver 11βHSD had no affinity for the same column (no 11βHSD activity eluted with any of the pyridine nucleotides; data not shown).

Human placental 11βHSD fractions that eluted from the column were analyzed by SDS-PAGE (Fig. 4C). A protein of 40,000 mol wt coeluted with 11βHSD activity (Fig. 4C). In multiple fractions over many chromatography experiments (under a variety of conditions), the 40K protein band, and no other, repeatedly segregated with 11βHSD activity (as shown in Fig. 4, C and D). This contrasts with the rat liver 11βHSD isoform that has a mol wt of 34,000 (3). The quantity of protein in the fractions with 11βHSD activity was estimated by comparison with known quantities of protein standards run in parallel. The purification achieved was in excess of 1000-fold (Fig. 4C).

When gradients of nucleotide cofactors were used to elute bound human placental 11βHSD from the AMP affinity column, the 11βHSD activity began to elute at thresholds of 30 μM NAD and 300 μM AMP, but did not elute at all during a 0- to 3000-μM NADP gradient (Fig. 4B). After the AMP gradient, a small amount of enzyme eluted with 3 mM NAD. After the NADP gradient, a large amount of 11βHSD activity eluted with NAD (Fig. 4B).

Substrate and cofactor preference of partially purified human placental 11βHSD

CHAPS produced competitive inhibition of human placental 11βHSD, reaching 50% (K_i) at 0.37 mM. Estimates of kinetic parameters for the partially purified enzyme eluted both with and without CHAPS were in good agreement when corrected for this competitive inhibition. Thus, the K_m of the partially purified human placental 11βHSD was 14 ± 1 nM (mean ± SE) for corticosterone and 54 ± 14 nM for cortisol, with a maximum velocity of approximately 25 nM/mg protein·min with either steroid. The cofactor preference of placental 11βHSD was examined by removal of NAD by repeated filtration in a Centricon-10 apparatus. At a residual NAD concentration below 2 μM, the addition of 400 μM NADP promoted an increase in 11βHSD activity. When

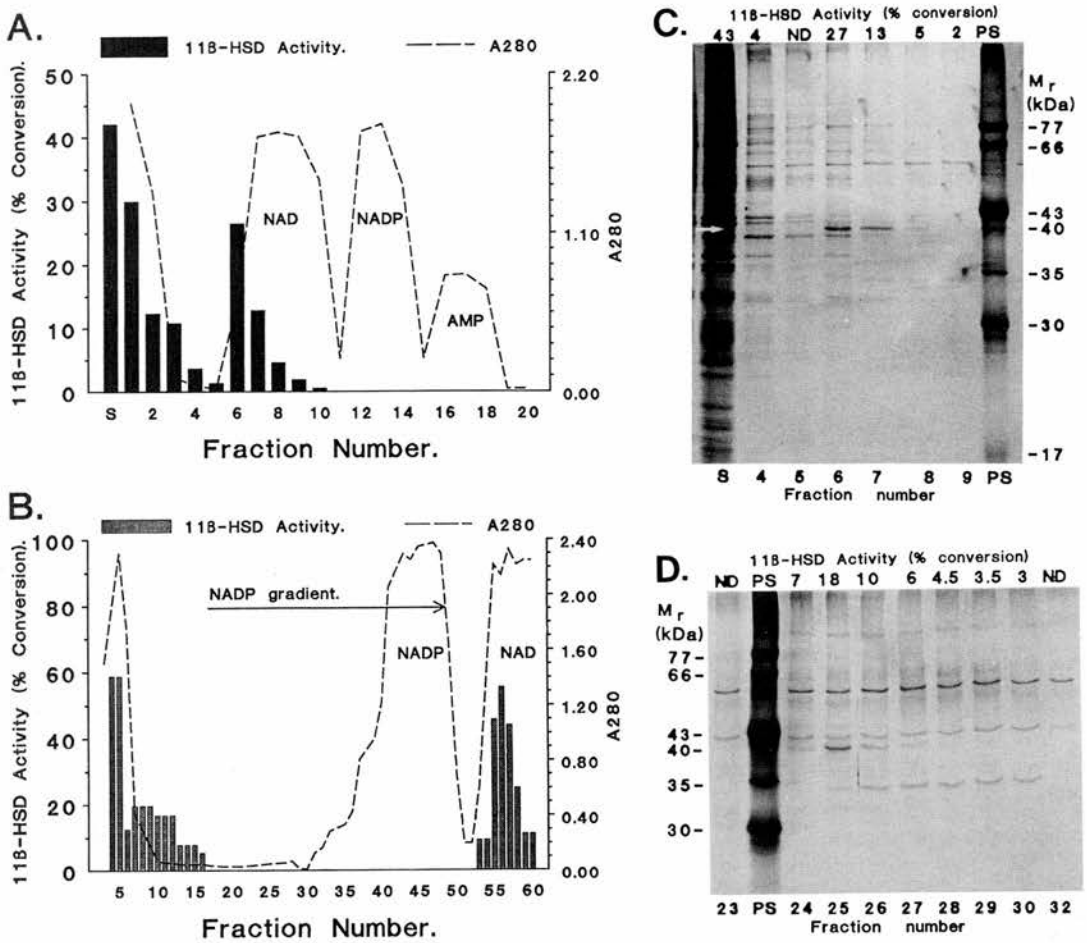

FIG. 4. AMP affinity chromatography of solubilized human placental 11βHSD. A and B, 11βHSD activity of fractions assayed with corticosterone and 400 μM NAD added. Spectrophotometric A_{280} demonstrates the falling protein concentration and shows cofactor addition. A: Fraction S Detergent-solubilized tissue extract loaded; fractions 1 and 2, flow-through/wash; fractions 3–5, wash; fractions 6–20, alternating cofactor elutions (as indicated) and brief washes. B: NADP gradient. Fractions 1–5, Flow-through/wash; fractions 6–15, wash; fractions 16–48, increasing NADP gradient (0–3000 μM); fractions 48–50, wash; fractions 51–60, 3 mM NAD elution. In the equivalent scheme for NAD and AMP gradients, 11βHSD eluted beginning at fractions 29 (30 μM) and 38 (300 μM), respectively (not shown). C and D, 12.5% SDS-PAGE gels (silver stained). C, Gel of the experiment shown in A. Proteins from 25 μl fraction S and 500 μl fractions 4–9 were loaded onto respective lanes. Corresponding 11βHSD activity (shown *above* gel) was assayed with corticosterone and 400 μM NAD and used 15 μl of each fraction. ND, Not detectable. PS, Protein standards which included the addition of 100 ng malate dehydrogenase [35K mol wt (M_r)]. The protein that repeatedly segregated with 11βHSD activity migrated at the position indicated by the *arrow* (40K mol wt). The factors of purification of 11βHSD in lanes 4, 6, 7, and 8 compared to that o fraction S were 40–50, greater than 1000, greater than 1000, and greater than 600, respectively. D, Gel showing proteins and 11βHSD activity tha eluted with NAD (beginning at lane 24) after a long wash. The 40K putative 11βHSD is the only protein consistently segregating with 11βHSD activity.

residual NAD was above 2 μM, the addition of 400 μM NADP was without effect. Thus, a more than 200-fold excess of NADP over NAD was required to demonstrate an effect on the enzyme.

Discussion

We have compared human placental, rat placental, and rat liver 11βHSD and found that the placental enzymes differ markedly from the rat liver isoform [the only isoform for which a cDNA has been isolated (4) and the gene cloned (30, 31)]. Placental 11βHSD differs from liver 11βHSD in cofactor preference, pH profile and optimum, latency release, and ease of solubilization in detergents. Moreover, we have achieved in excess of 1000-fold purification of human pla cental 11βHSD with AMP affinity chromatography (which was completely without affinity for rat liver 11βHSD). This is the first reported substantial purification of this enzyme Binding of human placental 11βHSD activity to the column has the properties expected for the active site of an NAD dependent enzyme. Mosbach and Brodelius (26, 27) have shown a linear correlation between thresholds for elution in affinity chromatography and the dissociation constants (K_d for the corresponding binary enzyme-nucleotide complexes Accordingly, the K_d and K_m of placental 11βHSD are pre dicted to be an order of magnitude or more higher for NADP than for NAD. This is strongly supported by the NAD preference of the enzyme both before solubilization and afte

affinity chromatography (when 400 μM NADP is without effect in the presence of NAD concentrations in excess of 2 μM). Human placental 11βHSD activity consistently segregates with a protein of apparent 40K mol wt, which we believe to be the strongest candidate to be the human placental 11βHSD enzyme [in contrast to an apparent mol wt of 34K determined by Lakshmi and Monder (3) on gels of identical composition for the purified rat liver isoform]. Although there are copurifying contaminating proteins, these vary and do not consistently segregate with 11βHSD activity. Human placental 11βHSD has a very high affinity for glucocorticoids, with a K_m of 54 nM for cortisol and 14 nM for corticosterone. Apparent K_m values, for the same substrates, of the unpurified enzyme (in the 25,000 × *g* pellet) were also estimated and found to be even lower. Liver 11βHSD has a much lower affinity for glucocorticoids, with a K_m over 100-fold higher for both corticosterone and cortisol (3, 5).

Rat placental 11βHSD seemed less abundant and more labile than its human counterpart, being easily inactivated by freeze/thaw or detergent action. However, despite this, the characteristics of 11βHSD in rat and human placentae seem qualitatively similiar; both have NAD preference, pH optimum between 7–8.5, poor solubility in active form in detergents, and relatively little activity with no added cofactor. The lability and poor solubility of rat placental 11βHSD precluded affinity chromatography.

Placental and rat liver 11βHSD seem to differ in some further characteristics. Firstly, the level of 11βHSD reductase activity in human term placenta is small compared to that of 11βHSD dehydrogenase activity, which is in contrast with the abundant rat liver 11βHSD reductase activity (5, 34). Secondly, the subcellular localization of 11βHSD appears different. Rat liver 11βHSD is localized to microsomal and nuclear fractions (34), and NADP-dependent rat kidney 11βHSD appeared to be similiarly distributed, with no activity in mitochondrial fractions (35). By contrast, our preliminary work suggests a different and more widespread subcellular localization of placental 11βHSD; further differential centrifugation produced mitochondria-enriched and microsome-enriched subfractions with similiar 11βHSD activities. Thirdly, rat liver microsomes were found to have substantial 11βHSD activity in the absence of added cofactor, being increased less than 2-fold by 400 μM NADP. In contrast, placental 25,000 × *g* membrane pellets had little 11βHSD activity in the absence of added cofactor, but this was increased 10-fold by 400 μM NAD. The cause of this difference is unclear, although the higher pyridine nucleotide cofactor concentrations in liver (32) and the relatively low K_m of liver 11βHSD for NADP (0.196 μM) (3) would cause any NADP sequestered in the microsomes to have a potent influence. The apparent K_m of human placental 11βHSD for NAD is considerably higher (13 μM), making the enzyme less sensitive to trace amounts of endogenous cofactors and causing it to show a clearer cofactor preference. In intact cells, NAD is more abundant (32), so both enzymes are suited to respond to physiological cofactor levels. Fourthly, our preliminary data suggest that their immunoreactivities to antiliver-type antiserum is very different. In conditions that cause minor immunoneutralization and major immunoprecipitation of solubilized liver microsomal 11βHSD, the solubilized placental 11βHSD showed absolutely no immunoneutralization or immunoprecipitation of 11βHSD activity with either 56–125 or 56–126 antiserum (10) (gift of Dr Carl Monder, Population Council, New York, NY).

Blasco *et al.* (19) calculated that an average human placenta approaching term is exposed to approximately 70 nmol maternal free cortisol/min. We estimate placental capacity to convert cortisol to cortisone to be at least 4 times higher than this. Moreover, the especially high affinity of placental 11βHSD for glucocorticoids would ensure their effective clearance, preventing access to the fetal circulation. Liver-type 11βHSD, although present in great abundance, would be relatively poorly suited to providing such a protective barrier, as its affinity for glucocorticoids is approximately 100-fold lower (K_m, 17 μM for cortisol and 1.8 μM for corticosterone).

The existence of a second enzyme (11βHSD2) has been the subject of much speculation (13), largely based on evidence of the presence of 11βHSD activity in the absence of liver-type 11βHSD protein or mRNA. In addition, variant mRNAs that hybridize to rat liver 11βHSD cDNA have been identified in kidney (8) and colon (9, 11). Further experimentation, however, has shown these renal mRNA variants to be derived from differential promoter usage of the characterized liver 11βHSD gene (31) and may encode proteins with no demonstrable 11βHSD activity (36). This has implications for *in situ* hybridization results on renal cortex (37, 38) as hybridization to liver-type 11βHSD probes in kidney may be detecting variant mRNA species without the potential for translation to active 11βHSD enzyme. Antibodies to liver-type 11βHSD also detect what appear to be additional tissue-specific proteins (*e.g.* 26K in brain, 40K in kidney, and 47K in testis) with strong antigenic similarities to liver-type 11βHSD (10). Indeed, when attempts have been made to separate such proteins (26K species) from the 34K liver-type 11βHSD protein, the variant protein had no demonstrable activity (12). Thus, it is possible that 11βHSD2 is the product of a separate gene and is not, therefore, readily detectable by antibodies or nucleic acid probes specific for liver-type 11βHSD. Both the NAD-dependent isoform in renal DCT (14–16) and the placental isoform described here may represent 11βHSD enzymes distinct from rat liver 11βHSD. It may be more than coincidence that both renal DCT and placental 11βHSD 1) are NAD dependent, 2) have a K_m for physiological glucocorticoids 2 orders of magnitude lower than that of liver 11βHSD (14), and 3) appear not to be immunoreactive to liver-type antiserum (6), and 4) that rat renal DCT activity is markedly inactivated by freeze/thaw (39), as we find for rat placental 11βHSD. If the rat renal DCT enzyme behaves like rat placental 11βHSD in detergents, it might explain why Triton X-100-solublized rat renal 11βHSD appeared to consist of the liver-type 11βHSD only (33). Moreover, a recent study of the 11βHSD cofactor preference in a range of tissues suggests that NAD-using 11βHSD activity is distributed more widely, being present in colon and lung as well as placenta and renal cortex (40).

In summary, we found strong evidence for an NAD-dependent 11βHSD isoform in both human and rat placental tissue that is distinct from the previously characterized enzyme in rat liver and may be encoded by a separate gene.

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EDITORIAL REVIEW

11-Beta-hydroxysteroid dehydrogenase: on several roads to hypertension

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Introduction

Elevated levels of adrenocorticosteroids (glucocorticoids or mineralocorticoids), due to Cushing's disease, Conn's syndrome or pharmacotherapy, are well-recognized causes of (secondary) hypertension. In essential hypertension blood steroid concentrations are usually normal, suggesting that excessive corticosteroid secretion is not causative in essential hypertension. It has recently become apparent that the effects of glucocorticoids at the tissue level are regulated by the presence of 11β-hydroxysteroid dehydrogenase (11β-HSD), which is crucial in determining glucocorticoid access to intracellular receptors. Thus, a novel level of control of corticosteroid action has been revealed, prompting a re-evaluation of the effective intracellular glucocorticoid concentration in particular tissues in various physiological and disease states (for reviews [1–6]). The present paper reviews the biology of 11β-HSD and its possible relevance to hypertensive disorders.

Syndrome of apparent mineralocorticoid excess and liquorice hypertension

11β-HSD catalyses the oxidation (ostensibly reversible) of the physiological glucocorticoids cortisol and corticosterone to their inactive 11-keto products (cortisone and 11-dehydrocorticosterone) [7]. Until recently the functions of this widespread enzymatic activity were obscure. However, deficiency of 11β-HSD had been noted in a rare type of hypertension known as the syndrome of apparent mineralocorticoid excess [8]. This disorder presents in childhood with sodium retention, severe hypertension and hypokalaemia. Plasma renin activity is undetectable but plasma levels of aldosterone and other mineralocorticoids are suppressed. In 1985 the investigation of a unique adult patient showed that the disorder was due to novel renal mineralocorticoid actions of cortisol [9]. Thus, the apparent renal mineralocorticoid excess and hypertension could be reversed by suppressing endogenous cortisol with dexamethasone, and recreated by concurrent infusion of physiological doses of cortisol. How could cortisol act as a potent mineralocorticoid?

Purified or recombinant mineralocorticoid receptors (type I corticosteroid receptors) are non-selective *in vitro*, and bind corticosterone, cortisol and aldosterone with similarly high affinities [10–12]. By contrast, mineralocorticoid receptors in the distal nephron are selectively activated by aldosterone *in vivo*, despite a 1000-fold molar excess of circulating glucocorticoid. Combining these paradoxical affinities and the findings in the syndrome of apparent mineralocorticoid excess led to the suggestion that in the distal nephron *in vivo* 11β-HSD eradicates cortisol, thus allowing selective access of aldosterone to mineralocorticoid receptors [9]. In the syndrome of apparent mineralocorticoid excess 11β-HSD deficiency leads to illicit binding of mineralocorticoid receptors by cortisol and, thus, apparent mineralocorticoid excess (Cushing's disease of the kidney [13]). It must be emphasized that circulating levels of cortisol are normal in the syndrome; it is the ability of the glucocorticoid to gain access to intracellular mineralocorticoid receptors in the distal nephron that

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is increased dramatically. Obviously, renal 11β-HSD must be very active; indeed, more than 99% of corticosterone is inactivated during its passage through a monolayer of cultured renal cortical collecting duct cells [14].

Supporting evidence for this hypothesis came from the imaginative recognition of the similarities between the syndrome of apparent mineralocorticoid excess and the hypertension observed with liquorice abuse [15]. Liquorice hypertension is the same type of hypertension as mineralocorticoid excess but requires intact adrenal glands and is reversed by dexamethasone. Moreover, liquorice, its active component glycyrrhetinic acid and the hemisuccinate derivative carbenoxolone are all very potent inhibitors of 11β-HSD *in vitro* and *in vivo* (their half-maximal inhibitory concentrations being in the nanomolar range) [15,16]. Indeed, administration of liquorice derivatives to rats and humans allows novel access of glucocorticoids to renal mineralocorticoid receptors *in vivo*, with consequent sodium retention, hypertension and hypokalaemia [15–19]. Although glycyrrhetinic acid and carbenoxolone inhibit a variety of other enzymes *in vitro* (including some which might lead to sodium retention [20–23], these actions require levels of the inhibitor that are unlikely to be achieved *in vivo* [24]. Moreover, the overwhelming metabolism of glucocorticoids in the distal nephron to their 11-keto products, rather than to other compounds [14], indicates that the apparent mineralocorticoid action of these inhibitors in man is predominantly due to inhibition of 11β-HSD.

Other actions of 11β-HSD

In addition to protecting mineralocorticoid receptors from exposure to cortisol in the distal nephron, placental 11β-HSD protects the foetus from the deleterious effects of maternal glucocorticoids ([25,26] and see below), 11β-HSD activity is also found in a wide variety of non-aldosterone-selective tissues, in which it might modulate functions mediated via type II glucocorticoid receptors [27,28]. Thus, topical hydrocortisone-mediated cutaneous vasoconstriction is potentiated by co-application of glycyrrhetinic acid [29] and, in the colon, control of Na,K-ATPase expression, mediated by type II glucocorticoid receptors, is potentiated by carbenoxolone [30]. Similar actions of 11β-HSD underlie the altered sensitivity to glucocorticoids during prenatal lung maturation [31] and in the pubescent testis [32]. Of course, the action of 11β-HSD is potentially reversible and the enzyme acts as a reductase (activating cortisone) in some tissues, such as adult liver [33–35], thereby recycling inert glucocorticoids [2].

Biochemistry and molecular biology of 11β-hydroxysteroid dehydrogenase

11β-HSD has been purified from rat liver and antisera have been raised against it [7,36]. The enzyme is reversible (acting either as a dehydrogenase, inactivating cortisol, or as a reductase, activating cortisone), nicotinamide adenine dinucleotide phosphate-dependent, predominantly located in microsomes and has an apparent molecular weight of 34 000 [7]. The purified enzyme has a Michaelis–Menten constant for cortisol of approximately 17 μmol/l and of 2 μmol/l for corticosterone [7,37,38]. Isolation and cloning of a complementary DNA (cDNA) species from a rat liver cDNA library [39] (and of its human homologue [40]) allowed expression of a single protein catalysing both dehydrogenation and reduction, the predominant direction being influenced, at least in cell culture, by cellular redox conditions and glycosylation status of the enzyme [41].

However, several lines of evidence suggest that 11β-HSD derived from the liver (hereafter 11β-HSD_1) is not sufficient to explain the many biological functions attributed to 11β-HSD. First, mineralocorticoid receptors and high 11β-HSD activity are found in the distal nephron [14,42–45], which is devoid of 11β-HSD_1 immunoreactivity [17,46]. Secondly, the micromolecular affinity of 11β-HSD_1 for cortisol and corticosterone [7] suggest that this enzyme would not exclude low (nanomolar) levels of circulating free glucocorticoids efficiently from renal mineralocorticoid receptors. Thirdly, the expression of 11β-HSD_1 cDNA in amphibian cells with a mineralocorticoid phenotype encodes an enzyme that acts predominantly as a reductase; far from inactivating corticosterone, it activates inert glucocorticoids [47]. A monkey 11β-HSD_1 homologue also acts as a reductase [48]. Fourthly, specific hormonal manipulations or developmental stages are associated with high renal 11β-HSD activity but low or undetectable 11β-HSD_1 messenger RNA expression [49–51]. Fifthly, syndrome of apparent mineralocorticoid excess patients have isolated dehydrogenase [9], but not reductase, deficiency and have no apparent mutations of the 11β-HSD_1 gene [52]. A distinct feature (hirsutism) is found in patients with the rare condition of isolated cortisone reductase deficiency [53]. Thus, the existence of several species of 11β-HSD has been postulated [1,6].

A family of 11β-hydroxysteroid dehydrogenases?

A shorter messenger RNA species (11β-HSD_{1b}) has been found [54,55], predominantly in kidney, which is the product of transcriptional initiation within

the first intron of the 11β-HSD_1 gene. However, although the putative protein product of this messenger RNA includes the potential active sites of 11β-HSD_1, there is little evidence that the messenger RNA for 11β-HSD_{1b} is translated *in vivo* and, when its cDNA is expressed *in vitro*, the recombinant protein is devoid of 11β-HSD activity, at least in the cells in which this was studied [56,57]. Why tissue-specific alternative transcripts without apparent biological function should be produced remains unexplained, although this might prevent any reductase activity of the almost ubiquitously expressed 11β-HSD_1 from jeopardizing the cortisol-free environment of mineralocorticoid receptors in the distal nephron.

More-convincing evidence for the existence of other 11β-HSD species followed the observation that C_{11}-dehydrogenation of some steroids was dependent on nicotinamide adenine dinucleotide, not on nicotinamide adenine dinucleotide phosphate, specifically in the distal nephron [58] and the placenta [59]. Recent data [60–62] have extended these observations and provide compelling evidence for a distinct nicotinamide adenine dinucleotide-dependent 11β-HSD (11β-HSD_2) in human placenta [60], rat kidney [61] and rabbit cortical collecting duct [62].

Placental 11β-HSD_2 differs from 11β-HSD_1 in pH optima, detergent solubility and lability, subcellular localization and immunoreactivity [60]. Substantial purification of this enzyme has been achieved, using affinity columns which do not retain 11β-HSD_1, revealing a protein of molecular weight approximately 40 000. Most importantly, placental 11β-HSD_2 has a much higher affinity for cortisol and corticosterone (with Michaelis–Menten constants in the low nanomolar range) and exhibits little or no reductase activity [60]. The nicotinamide adenine dinucleotide-dependent 11β-HSD in the distal nephron also has high affinity for glucocorticoids [62], although it is not clear whether this species of 11β-HSD is identical to placental 11β-HSD_2. Whether further enzymes possess 11β-HSD-like activity is not known but, intriguingly, no genetic defect of the 11β-HSD_1 gene was found in a patient with isolated reductase deficiency [52], implying the existence of a third gene encoding an 11β-HSD species which acts predominantly as a reductase.

From the above it is apparent that current understanding of the molecular biology and biochemistry of the important 11β-HSD enzyme family is far from complete. Nevertheless, many studies of enzyme function have been undertaken, and evidence for the possible roles of different 11β-HSD species in the pathogenesis of hypertension is reviewed below.

11β-Hydroxysteroid dehydrogenase and renal (mineralocorticoid) hypertension

Deficient activity of 11β-HSD might be expected to cause exclusively mineralocorticoid hypertension, but many other blood pressure-related actions of 11β-HSD have been postulated. In addition to the rare examples of syndrome of apparent mineralocorticoid excess and liquorice abuse, attenuation of 11β-HSD-mediated protection of renal mineralocorticoid receptors could also account for the hypertension and hypokalaemia seen in the ectopic adrenocorticotrophic hormone syndrome, which is likely to reflect 11β-HSD inhibition, rather than saturation of the enzyme by cortisol excess [63]. Whether adrenocorticotrophic hormone affects renal 11β-HSD directly, or indirectly inhibits the enzyme via induction of alternative substrates [5], is not clear. In renal failure 11β-HSD activity is attenuated, as determined by reduced cortisol metabolism at the 11α position [3], but so is expression and responsiveness of renal mineralocorticoid receptors, thus complicating interpretation.

A proportion of essential hypertensive patients (perhaps one-third) have a prolonged plasma half-life of 11α-[^{3}H]-cortisol, suggesting 11β-HSD deficiency [64,65]. These individuals show no signs of mineralocorticoid excess and, intriguingly, may also show attenuated 11β-reductase activity [64], suggesting the involvement of 11β-HSD_1 since reductase activity is, at most, a minor feature of 11β-HSD_2 catalysis [60,62], but is a prominent activity of 11β-HSD_1 [7,41,47].

11β-Hydroxysteroid dehydrogenase and vascular effects of glucocorticoids

11β-HSD_1 is found in resistance vessels, conducting vessels and the heart [66–68] in which tissues the enzyme might modulate glucocorticoid-mediated vascular responses [64], including potentiation of vasoconstriction to noradrenaline [69]. Indeed, in congenital and liquorice-induced 11β-HSD deficiency the vasoconstrictive potency of cortisol is increased [70]. Increased dermal vascular sensitivity to glucocorticoids is observed in essential hypertension, suggesting that endogenous cortisol might have easier access to mineralocorticoid receptors in hypertension [71]. However, the increased sensitivity to cortisol-induced vasoconstriction is found both in patients with a prolonged and in those with a normal half-life of 11α-[^{3}H]-cortisol [64]. Furthermore, in essential hypertension, but not in congenital or acquired 11β-HSD deficiency, dermal vascular sensitivity to a synthetic glucocorticoid (beclomethasone; thought to be a poor substrate for 11β-HSD) is also

increased [71]. The molecular basis and importance of this increased vasoconstrictor response to glucocorticoids in essential hypertension remains to be investigated. However, unless the vascular activity (due to 11β-HSD_1 [68]) can metabolize beclomethasone, vascular deficiency of 11β-HSD is unlikely to be involved in essential hypertension.

Central hypertensive effects of corticosteroids and 11β-hydroxysteroid dehydrogenase in the brain

Corticosteroids exert specific central effects on blood pressure in the rat. Thus, intracerebroventricular administration of aldosterone in small (nanogram) doses, which are ineffective when given peripherally, increases blood pressure [72]. Intracerebroventricular corticosterone antagonizes the aldosterone effect [73], whereas intracerebroventricular glycyrrhetinic acid also elevates blood pressure, suggesting that a population of central aldosterone-selective mineralocorticoid receptors protected by 11β-HSD might mediate the central hypertensive effects of aldosterone and glycyrrhetinic acid. Indeed, some brain subregions bind aldosterone selectively *in vivo* [74,75]. 11β-HSD_1-like bioactivity and expression of the messenger RNA is widespread in the rat brain [28,76–79], predominantly in neurons [28,80]. 11β-HSD_2 may also be present in the hypothalamus [81], which is a likely site for aldosterone-selective central actions on blood pressure [82]. Furthermore, glycyrrhetinic acid administration increases neuronal activity selectively in the anterior hypothalamus, reinforcing the notion that important 11β-HSD-related effects occur at this site [83]. Although these data suggest that protection by 11β-HSD_2 of mineralocorticoid receptors in the brain from glucocorticoids is important in blood pressure regulation, it is possible that much of the 11β-HSD_1 in the brain functions predominantly as reductase [81]. This raises the possibility that inhibition of 11β-HSD might cause regional reductions in glucocorticoid supply to corticosteroid receptors in the brain, including those glucocorticoid receptors which normally antagonize mineralocorticoid receptor-mediated hypertensive effects. Clearly, further studies are required to define the anatomy and physiology of these potentially important functions of 11β-HSD.

Placental 11β-hydroxysteroid dehydrogenase and the programming of hypertension

Extensive epidemiological data [84,85] have recently implicated prenatal events as potent determinants of subsequent blood pressure level (and the risk of death related to cardiovascular disease). In particular, low (non-premature) birth weight (and, perhaps, a large placenta) strongly predicts higher blood pressure in childhood, adolescence and adulthood, this correlation being apparently independent of adult risk factors, such as smoking, alcohol consumption, weight or socio-economic status [84,85]. Several possible explanations for these findings have been proposed, concentrating largely on the deleterious effects of abnormal maternal nutrition [84,85]. However, glucocorticoid administration to the pregnant mother also reduces birth weight in animals and in man, and glucocorticoids increase blood pressure both in fetal and in adult animals [86,87]. The unusual combination of low birth weight and increased placental weight is also found in pregnant rats with streptozotocin-induced diabetes; the fetal and placental abnormalities are only partly reversed by insulin treatment producing normoglycaemia [88], and such rats also have markedly elevated glucocorticoid levels at term [89]. Although exogenous glucocorticoid treatment usually reduces placental weight, this does not always occur with low doses of physiological glucocorticoids [90]. Furthermore, higher adult blood pressures are also observed in some humans with low birth weight and small placentae [84,85].

What effect does intra-uterine glucocorticoid exposure have on the blood pressure of the offspring? Administration of dexamethasone (which crosses the placenta freely [91]) to pregnant rats, in a modest dose which reduces birth weight by only 14%, leads to substantially higher blood pressures in the offspring when they reach maturity, more than 5 months after the last exposure to exogenous glucocorticoid [92]. The elevation of blood pressure persists and is seen in old rats (Lindsay R, Noble J, Edwards C, Seckl J: unpublished observations, 1993). Whether there is a particular developmental 'window of sensitivity' to glucocorticoid programming of elevated blood pressure in the rat is not clear, although this may occur in fetal sheep during the second trimester [87].

The mechanism of hypertension in rats exposed to dexamethasone *in utero* is unknown, but is likely to reflect permanent programming of blood pressure which may then track with age [93]. Secondary changes may then amplify the phenotype. Relatively minor stresses in the neonatal period programme expression of corticosteroid receptors in limbic areas of the brain throughout the lifespan of a rat [94]; preliminary data suggest that exposure to dexamethasone *in utero* has similar effects in certain brain regions in the rat (Holmes M, Lindsay R, Seckl J: unpublished observations, 1993) [95]. Many other actions of prenatal exposure to glucocorticoids can also be envisaged, including alterations in microvascular development; potentiated cardiovascular responses to pressor substances, especially cate-

cholamines (as seen after low-dose dexamethasone treatment of fetal rats in late gestation [96]) or angiotensin II (observed with infusion of cortisol in fetal sheep [87]); altered development of sympathetic innervations or expression of adrenergic receptors in target organs (seen in the fetal lung [97] or neonatal liver [98] after dexamethasone administration to pregnant rats); and changes in the synthesis, transport or action of growth factors on fetal organs or the placenta, or both [99]. Finally, indirect actions of dexamethasone on maternal cardiovascular dynamics or ability to support fetal and placental growth could be involved, although maternal electrolyte imbalance and plasma volume expansion do not appear to occur at the dose of glucocorticoid used (Lindsay R, Seckl J: unpublished observations, 1993).

Normally, of course, placental 11β-HSD_2 excludes maternal glucocorticoids from the fetal circulation, at least later in gestation [26,100]. Intriguingly, placental 11β-HSD activity at term in the rat is lowest, and hence fetal exposure to glucocorticoids is greatest, in the smallest foetuses with the largest placentae [92], that is, those expected, on the basis of studies in humans, to exhibit the highest blood pressures in adulthood [101]. Placental 11β-HSD deficiency may thus allow maternal glucocorticoids access to the foetus, retarding its growth and programming or imprinting patterns of responses which lead to subsequent hypertension [95]. It will be important to determine whether prolonged administration of synthetic glucorticoids to pregnant women affects the blood pressure of their offspring, since glucocorticoids that are poor substrates for 11β-HSD (e.g. dexamethasone) currently are more widely used in the prenatal therapy of 21-hydroxylase deficiency and other congenital adrenal hyperplasia syndromes (prednisolone is subject to substantial metabolization by placental 11β-HSD [91]). Also, there may be distinct windows for glucocorticoid actions on the foetus, since at mid-gestation, at least in primates and humans, reductase activity appears to dominate in the placenta [34,59], suggesting that 11β-HSD_1 expression precedes that of 11β-HSD_2. The implications of excessive or deficient activation of cortisone by placental reductase at mid-gestation have not yet been studied.

11β-Hydroxysteroid dehydrogenase and other hypertensive mechanisms

Other glucocorticoid target organs may employ 11β-HSD-mediated mechanisms relevant to blood pressure control. Prominent among these may be the liver, which has both high 11β-HSD activity and high glucocorticoid receptor density. Indeed, in the Bianchi–Milan rat model of hypertension it is hepatic, rather than renal, 11β-HSD activity that is altered (reduced) [102]. Intriguingly, changes are seen in young prehypertensive rats, suggesting a possible pathogenetic importance of 11β-HSD in hypertension. However, no clear functions of hepatic 11β-HSD have been defined; although modulation of glucocorticoid effects on insulin sensitivity, insulin-like growth factor synthesis or angiotensinogen production are potential functions, they await study. The predominant direction of the reaction catalysed by 11β-HSD within hepatic subregions has also not been determined, although reduction (activation of cortisone) appears to be predominant in the liver overall [19,33–35]. Indeed, the grossly elevated (total) plasma corticosterone levels in Bianchi hypertensive rats may, at least partly, reflect the lack of intrahepatic activation of glucocorticoids by 11β-HSD and the consequent increase in corticosteroid-binding globulin synthesis, itself negatively regulated by glucocorticoids in adult rats [103].

The pituitary gland and hypothalamus also express 11β-HSD [78], and might modulate activity of the hypothalamic–pituitary–adrenal axis [104] or of growth hormone release, affecting tissue growth and blood pressure regulation. Finally, adipose tissue also exhibits 11β-HSD activity [105], and glucocorticoids exert well-documented actions on fat distribution and metabolism. The centripetal obesity of Cushing's syndrome and syndrome X, both associated with hypertension, might be modulated by variations in adipocyte 11β-HSD. Indeed, syndrome X is very accurately predicted by low birth weight [106], providing a possible link between deficient placental 11β-HSD and disorders other than hypertension.

In summary, different species of 11β-HSD are likely to regulate many biological processes mediated by glucocorticoids. Tissue-specific defects in 11β-HSD expression are clearly associated with rare congenital and acquired hypertensive syndromes. In the more common condition of essential hypertension, the importance of these enzymes requires clarification; particularly lacking is a clear understanding of the direction of enzyme activity in specific target tissues *in vivo*. Dysfunction of placental 11β-HSD_2 may provide a crucial link between retardation of intra-uterine growth and the attendant substantial risk of common cardiovascular and metabolic disorders, particularly hypertension, in later life.

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Purification of 11β-hydroxysteroid dehydrogenase type 2 from human placenta utilizing a novel affinity labelling technique

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11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) efficiently inactivates potent glucocorticoid hormones (cortisol and corticosterone), leaving aldosterone unmetabolized. Abundant 11β-HSD2 activity in human placenta plays a central role in controlling fetal glucocorticoid exposure, which if excessive is harmful and may predispose to low birth weight and hypertension in adulthood. Similar 11β-HSD2 activity in the distal nephron protects mineralocorticoid receptors from glucocorticoids and appears to be important in normal blood pressure control. We have purified human placental 11β-HSD2 16000-fold, to homogeneity, and determined over 100 residues of the internal amino acid sequence. Purification was assisted by a novel technique allowing highly specific (single spot on two-dimensional electrophoresis) photoaffinity labelling of active 11β-HSD2 in crude tissue extracts by its glucocorticoid substrates. This work reveals that 11β-HSD2 is a member of the short-chain alcohol dehydrogenase superfamily (apparent monomer $M_r \sim 40000$). It is a very basic (apparent pI = 9.1) intrinsic membrane protein, requiring as yet undefined membrane constituents for full stability. Affinity chromatography and affinity labelling studies suggest that 11β-HSD2 has a compulsory ordered mechanism, with NAD^+ binding first, followed by a conformational change allowing glucocorticoid binding with high affinity.

INTRODUCTION

11β-Hydroxysteroid dehydrogenase (11β-HSD) catalyses the rapid metabolism of physiological glucocorticoids (cortisol and corticosterone) to inactive 11-dehydro products (cortisone and 11-dehydrocorticosterone respectively). In any tissue the activity of 11β-HSD will affect the amount of circulating glucocorticoids reaching their intracellular receptors [glucocorticoid receptors (GR) and mineralocorticoid receptors (MR)]. Moreover, as the mineralocorticoid aldosterone is not metabolized by 11β-HSD, it can gain selective access to the MR in the face of 100–1000-fold higher levels of circulating glucocorticoids, providing that 11β-HSD activity is sufficient to eradicate the glucocorticoid. It is now widely believed that just such a selective barrier to glucocorticoid is present in aldosterone target tissues [1,2] (e.g. the distal nephron in kidney) and enables potent effects on fluid and electrolyte balance and blood pressure to be exerted by aldosterone acting through MR (which *in vitro* bind aldosterone and physiological glucocorticoids with equal affinity [3]). Deficiency of 11β-HSD due either to the congenital syndrome of apparent mineralocorticoid excess (SAME) [4,5] or to the ingestion of 11β-HSD inhibitors [6] (liquorice constituents or carbenoxolone) leads to the normally protected tissues being accessed by glucocorticoids, which occupy MR and cause mineralocorticoid hypertension.

There is also abundant 11β-HSD activity in the placenta which has a major influence on the glucocorticoid exposure of the developing fetus [7–10]. Glucocorticoids are important in normal development, facilitating the maturation of tissues (e.g. lung [11]) and influencing the set point of aspects of fetal physiology, a number of which then become fixed for life (fetal programming) [12–15]. If the fetus is exposed to excessive glucocorticoids, birth weight is reduced [9,16] and, in animal models, the offspring are hypertensive in adulthood [9]. Inhibition of placental 11β-HSD similarly reduces birth weight and results in hypertensive offspring (R. S. Lindsay, R. M. Lindsay, C. R. W. Edwards and J. R. Seckl, unpublished work). Human epidemiology also reveals that those babies with the lowest birth weight have the greatest risk of adult hypertension [17]. Furthermore, in rats [9] and humans [10], birth weight correlates with placental 11β-HSD activity.

The previously identified isoform of 11β-HSD (11β-HSD1) is not responsible for the renal and placental barriers to glucocorticoids. It has been purified from rat liver [18], and corresponding rat [19], human [20], squirrel monkey [21], sheep [22] and mouse [23,24] cDNA clones have been isolated. 11β-HSD1 is a reversible, NADP(H)-dependent enzyme, with high expression in some tissues (e.g. liver), but not in the distal nephron or placenta, and it has relatively low affinity for glucocorticoids (K_m in the low micomolar region) [18]. Moreover, no mutations of the 11β-HSD1 gene have been identified in SAME patients [25]. A distinct NAD^+-dependent, exclusively dehydrogenase, 11β-HSD activity with high affinity for glucocorticoids has been identified in biochemical studies in rabbit kidney cortical collecting duct cells [26] and human placenta [27]. Similar activity has now been described in renal tissue of other species [28] and several human fetal tissues [29]. These activities are due to a distinct isoform, 11β-HSD2 (or possibly several closely related ones), and seem likely to be responsible for the important barriers to glucocorticoid access described above. Further study of 11β-HSD2 has been hampered by the lack of specific 'molecular tools' to investigate it at the protein and nucleic acid levels.

In this paper we present the first purification of 11β-HSD2 to homogeneity and an extensive amino acid sequence derived directly therefrom. We also describe a novel affinity labelling procedure allowing 11β-HSD2 to be specifically labelled by its natural substrates. Aspects of this work should facilitate study of the mechanism of action of 11β-HSD2 and the mapping of its

Abbreviations used: 11β-HSD(2), 11β-hydroxysteroid dehydrogenase (type 2); GR, glucocorticoid receptors; MR, mineralocorticoid receptors; SAME, syndrome of apparent mineralocorticoid excess; CAPS, 3-(cyclohexylamino)-1-propanesulphonic acid; 2-D, two-dimensional; CMC, critical micelle concentration; IEF, isoelectric focusing; NEPHGE, non-equilibrium pH-gradient gel electrophoresis; TFA, trifluoroacetic acid; SCAD, short-chain alcohol dehydrogenase; PVDF, poly(vinylidene difluoride).

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active site. Such study will be valuable in the efficient design of new drugs influencing corticosteroid physiology (e.g. by selectively inhibiting, bypassing or being metabolized effectively by this key enzyme). In an accompanying paper we report use of the 11β-HSD2 amino acid sequence to isolate and characterize a full-length human placental 11β-HSD2 cDNA clone and to raise antisera to the placental 11β-HSD2 protein [30].

EXPERIMENTAL

Materials

[1,2,6,7-^{3}H]Corticosterone, [1,2,6,7-^{3}H]cortisol, [1,2,4,6,7-^{3}H] dexamethasone and [1,2,6,7-^{3}H]aldosterone (specific radioactivity 78, 73, 84 and 80 Ci/mmol respectively) were obtained from Amersham International (Little Chalfont, Bucks., U.K.). Autodigestion-resistant modified trypsin and *Staphylococcus aureus* V8 protease were obtained from Promega (Southampton, U.K.), and hydrogenated Triton X-100 (RTX-100; protein grade) was from Calbiochem (Nottingham, U.K.). Glycerol and protein standards for SDS/PAGE (#44264L), electrophoresis-grade SDS and urea were purchased from BDH Laboratory Supplies (Poole, Dorset, U.K.). Coomassie Blue dye concentrate, standardized BSA and protein standards for two-dimensional (2-D) electrophoresis (#1610320) were purchased from Bio-Rad (Hemel Hempstead, U.K.). HPLC-grade methanol and water were purchased from Rathburn Chemicals (Walkerburn, Scotland, U.K.) and Quickszint 302 HPLC scintillant was from Zinsser Analytic (Maidenhead, U.K.). Entensify fluoroautoradiography solutions were obtained from du Pont/NEN (Stevenage, U.K.). Poly(vinylidene difluoride) (PVDF; Problott) membranes were obtained from Applied Biosystems (Warrington, U.K.). Affinity chromatography matrices, Pharmalytes, detergents and other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Buffers

Chromatography buffer systems used were as follows. Buffer A: 20% glycerol, 5 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM dithiothreitol. Buffer B: 10% glycerol, 300 mM NaCl, 4 mM CHAPS, 1 mM EDTA, 0.02M Tris/HCl, pH 7.7. Buffer C: 10% glycerol, 300 mM NaCl, 1 mM EDTA, 0.02 M Tris/HCl, pH 7.7. Where the buffer varies from that above, the variation is appended in [brackets].

Subcellular fractionation of placental tissue

Human term placentae (400–600 g; normal vaginal delivery) were rapidly placed on ice, and tissue was processed within 2 h. Adherent membranes, umbilical cord and large vessels were removed. Placental tissues were rapidly minced with scissors, washed in ice-cold 0.9% NaCl, blotted dry, suspended in approximately 3 times their weight of buffer A and homogenized with a commercial blender. Homogenate was filtered through two layers of muslin and the filtrate fractionated rapidly by differential centrifugation: (i) 10 min at 750 g, (ii) 40 min at 25000 g and (iii) 60 min at 110000 g. The supernatant from each centrifugation was subjected to the next centrifugation, finally leaving a cytosolic supernatant. Resulting fractions were frozen at −80 °C or used immediately.

Assays of 11β-HSD activity

11β-HSD2 activity was determined by measuring the percentage conversion of 1.12×10^{-8} M ^{3}H-labelled steroid substrate (corticosterone unless otherwise stated) to product (11-dehydrocorticosterone) in the presence of NAD^+ (400 μM unless otherwise stated). The 250 μl assay consisted of 10 μl containing tritiated steroid, 50 μl containing cofactor and 190 μl of enzyme in buffer C. Reactions were incubated at 37 °C for 10 min and terminated by adding 2 ml of ethyl acetate. The steroids (in the organic layer) were assayed by HPLC as previously described [27], and the percentage conversion of steroid substrate to product was calculated as an index of enzyme activity. Protein was estimated by the method of Bradford [31]. 11β-HSD assay conditions were such that the amount of protein added was in the linear region of the curve of protein concentration versus percentage substrate converted with 400 μM NAD^+, and resulted in conversion of 10–40% in 10 min. All experiments had blank (no protein) assays run in parallel. Kinetic parameters were calculated from initial-velocity determinations in assays giving less than 30% conversion.

Detergent solubilization

All solubilization was carried out at 0–2 °C. Tissue fractions, resuspended in buffer C to 6 mg of protein/ml, were mixed with an equal volume of solubilization buffer (in buffer C with detergent at twice the final concentration). After 30 min the mixture was centrifuged at 110000 g for 1 h. Supernatant containing soluble enzyme was carefully removed. A screen was performed using 13 detergents of diverse classes [32] (Triton DF18 [18], CHAPS, taurodeoxycholic acid, polyoxyethylene-10-lauryl ether, digitonin, n-octyl glucoside, Tween 20, Tween 80, SDS, n-lauryl sarcosine, Triton X-100, Zwittergent 3-10 and Bigchap). Following this screen, conditions were refined for the most promising detergent (CHAPS).

Affinity chromatography

Affinity chromatography matrices were hydrated, loaded into simple columns and equilibrated in buffer B. For the preparative 5′-AMP–agarose chromatography, six 5 ml columns were run in parallel, having a common outflow; 5 ml fractions were collected. A CHAPS-solubilized placental 25000 g pellet fraction (180 ml) was loaded (2.4 ml/min) on to the columns, which were then washed with 205 ml of buffer B and 45 ml of buffer B[0.25 M NaCl], and 11β-HSD was eluted with 170 ml of buffer B[0.2 M NaCl + 1 mM NAD^+], all at 3.5 ml/min. Fractions were placed on ice and rapidly assayed for 11β-HSD enzyme activity. Fractions with abundant 11β-HSD2 were pooled and concentrated (Amicon stirred cells; Centricon 10 concentrators; acetone precipitation).

Analytical work used 1 ml columns run manually (1.0–1.5 ml fractions). To obtain the highest purity of eluted 11β-HSD an N-6-5′-AMP–agarose column was washed with buffer B (15 ml), buffer B[0.02 M NaCl + 0.4 mM NADH] (10 ml) and buffer B (15 ml), and then eluted with buffer B[0.125 M NaCl + 1 mM NAD^+]. The flow rate was 420 μl/min, decreasing to 200–300 μl/min on elution. The increased purity was at the cost of lower yield and reproducibility than with the preparative protocol (above).

UV photoaffinity labelling and fluoroautoradiography

A portion of 467 μl of tissue sample diluted in buffer C was added to wells of 24-well plates (diameter 15 mm) and maintained at 37 °C for 3 min. Addition of 60 μl of 250 mM dithiothreitol, 48 μl of 5 mM NAD^+ (both in buffer C) and finally [^{3}H]steroid (in 25 μl of 10% ethanol/buffer C) completed the reaction, giving approx. 50 nM [^{3}H]steroid in 600 μl, unless otherwise

stated. In the experiments presented here (to minimize UV damage to protein), reactions were placed in UV light (312 nm transilluminator at a distance of 50 mm above plate; lid off) for 15 min at 37 °C. The procedure was easily scaled up. Similar labelling occurs at 254 nm and is even stronger at 0 °C or with longer UV exposure. However, all three of these variations also induce formation of minor bands below M_r 40000, and protein damage with prolonged 254 nm UV exposure will be more extensive.

Labelled samples were acetone-precipitated and resolved by SDS/PAGE [33] or 2-D electrophoresis. Finished gels, stained with Coomassie Blue to allow detection of major proteins and M_r standards, were processed for fluoroautoradiography in Entensify solutions and vacuum-dried before exposure to film.

2-D electrophoresis

2-D electrophoresis work involved running duplicate gels in parallel; usually one was silver stained (to visualize all the proteins), and the other stained with Coomassie Blue (to allow alignment between gels and to visualize SDS/PAGE protein standards) and processed for autoradiography. A set of 2-D electrophoresis protein standards was run under identical conditions on a third gel when new running parameters were used.

Conventional 2-D PAGE, i.e. isoelectric focusing (IEF) plus SDS/PAGE, used a variation of the method of O'Farrell [34]. 2-D non-equilibrium pH-gradient gel electrophoresis (NEPHGE) conditions were based on the methods of O'Farrell [35] and Witzmann [36], with variations of sample buffer and rod gel composition. Rod gels (170 mm × 2.5 mm; length × internal diameter) were cast from 9 M urea, 2% Nonidet P40, 4% acrylamide/bisacrylamide (19:1, w/w) and 3% Pharmalytes (1.2% pH 6–8 and 1.8% pH 3–10), and polymerized with 32 μl of 10% ammonium persulphate and 27 μl of *NNN'N'*-tetramethylethylenediamine (TEMED) per 20 ml of rod gel mixture. Optimal results (higher yield, better resolution), especially for basic hydrophobic proteins, were obtained with a dodecyl maltoside-based NEPHGE sample buffer: 5.71 g of urea, 154 mg of dithiothreitol, 4 ml of 10% dodecyl maltoside, 750 μl of Pharmalytes (300 μl of pH 6–8; 450 μl of pH 3–10) and 2.25 ml of HPLC-grade water, and adjusting the pH to 4.2 (by adding approx. 90 μl of 5 M HCl and 160 μl of HPLC-grade water). This buffer, which was a great improvement, is close to solidifying at room temperature and is melted, by warming to 30 °C, to adjust pH on preparation and on use. NEPHGE gels were run at 500 V for 2250 V·h. After NEPHGE, rods were extruded into 10 ml of equilibration buffer for 7 min and either frozen or loaded directly on to second-dimension SDS/PAGE gels. Equilibration buffer (150 ml) consisted of 15 ml of β-mercaptoethanol, 9 g of SDS, 90 ml of HPLC-grade water, 5.6 ml of 0.075% Bromophenol Blue and 37.5 ml of 4× Laemmli stacking buffer (0.4% SDS, 0.5 M Tris/HCl, pH 6.8). For both IEF and NEPHGE the second dimension was run according to the resolving gel system of Laemmli [33] using 12.5% gels (3.3% cross-linker). Silver staining was by the method of Wray [37].

Concentrated protein from preparative AMP affinity chromatography runs was divided among several 1.5 mm-thick preparative 2-D gels. Precautions were taken to minimize protein N-terminal modifications: the second-dimension gel was pre-run in a Laemmli running buffer containing 0.5% (v/v) freshly made 100 mM glutathione and then changed to running buffer with 0.1% (v/v) 100 mM sodium thioglycholate [38]. These precautions were successful in allowing N-terminal sequence to be obtained for other proteins in our laboratory.

Protein blotting for sequencing

This was based on the CAPS [3-(cyclohexylamino)-1-propanesulphonic acid] buffer method of Matsudaira [39] and was performed from SDS/PAGE or second-dimension gels on to PVDF (Problott) membranes using a Bio-Rad Mini Trans-Blot apparatus. Sections of gels to be blotted were washed for exactly 30 s in CAPS transfer buffer (to allow shrinkage and removal of excess surface SDS). Electroblotting was for 75 min at 250 mA (90–110 V). After transfer, the PVDF sheets were stained (Amido Black), destained (water) and air dried, and desired bands/spots were cut out and stored at −70 °C.

Proteolytic digests of PVDF-blotted protein

These were based on the method of Fernandez et al. [40]. Membranes were cut into small pieces, blocked [0.2% polyvinylpyrrolidone (average M_r 40000) in methanol for 45 min] and extensively washed in deionized HPLC-grade water. Autodigestion-resistant trypsin was added in AHT buffer (20% acetonitrile, 1% hydrogenated Triton X-100 in 100 mM Tris, pH 8) to a concentration of 1:20 (w/w) (trypsin/blotted protein), and digestion was continued for 24 h at 37 °C. Fragments were eluted using vigorous sonication and washes [AHT buffer, then 0.1% (w/v) trifluoroacetic acid (TFA)] [40]. Eluted peptides were concentrated, removing the acetonitrile, and loaded as a 100 μl sample on to an Applied Biosystems microbore HPLC system fitted with a C8 RP300 column (1 mm × 250 mm; 7 μm particle size) equilibrated in solvent A (0.1% TFA/water). This was developed with a linear gradient of 0–100% solvent B (0.08% TFA in 80% acetonitrile/water) over 45 min at 200 μl/min. The spectrophotometric absorbance at 220 nm (A_{220}) of eluate allowed collection of peptide peaks. Control digests (trypsin with polyvinylpyrrolidone-blocked blank PVDF slices) were run in parallel to ensure identification of all peaks not due to the blotted protein substrate. A large peptide peak yielding no sequence which was thought to be the blocked N-terminal peptide was sub-digested in solution (50 mM sodium phosphate, pH 7.8) with *S. aureus* V8 protease at an estimated concentration of 20:1 (w/w, peptide/protease). Products were resolved on HPLC, as were those from a control (protease only) digest.

Amino acid sequencing

Peptide peaks selected for sequencing were pyridyl-ethylated [41], to allow detection of cysteine residues, and loaded into an Applied Biosystems 477A automated amino acid microsequencer.

RESULTS AND DISCUSSION

Subcellular fractionation

Human placental 11β-HSD activity was well preserved during subcellular fractionation. Placental 11β-HSD was clearly a membrane-associated protein (as < 2% of activity remained in the cytosolic supernatant) and was most abundant in the 25000 *g* pellet, which contained over two-thirds of the total enzyme activity in under one-sixth of the total protein. Differential centrifugation of the 25000 *g* pellet did not produce any useful refinement. This fraction was used for further purification, and has been found to be very stable on storage at −70 °C, with no decline in activity in over 2 years.

Solubilization

Solubilization (i.e. remaining in supernatant after 1 h at 110000 *g*) of placental 11β-HSD (from resuspended 25000 *g*

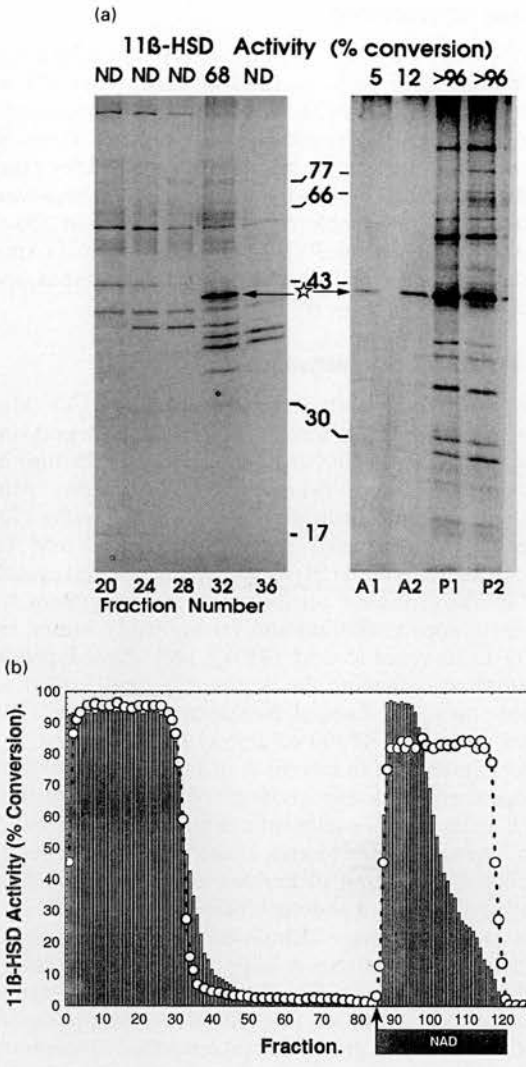

Figure 1 5′-AMP affinity chromatography

(**a**) SDS/12.5%-PAGE gels (silver stained) from 5′-AMP–agarose chromatography. 11β-HSD2 activity (percentage conversion of 12 nM [^{3}H]corticosterone in 10 min) is indicated above the lanes; ND, not detected. The positions of 11β-HSD2 protein (starred arrow) and of markers ($10^{-3} \times M_r$) are shown between the panels. Left panel, preliminary analytical study showing samples before, during and after harsh elution with NAD^+ (fractions 31–34); right panel, samples from two particularly clean analytical runs (A1 and A2) and pooled high-activity fractions from two typical preparative runs (P1 and P2), as described in the Experimental section. For analytical runs 190 μl samples of fractions were used in standard 11β-HSD assays and 1 ml samples (acetone precipitated) were used for SDS/PAGE; in preparative runs 50 μl (assays) and 250 μl (SDS/PAGE) samples were used. (**b**) Chromatographic profile of preparative-scale run showing 11β-HSD2 activity (bars) in relation to A_{280} (protein concentration or NAD^+ on elution) of fractions (5 ml) from the common outflow of six 5 ml AMP–agarose columns run in parallel. Fractions 1–36, loading; 37–86, washes; 87–120, elution with 1 mM NAD^+. The most active of these fractions are equivalent to samples P1 and P2 described above.

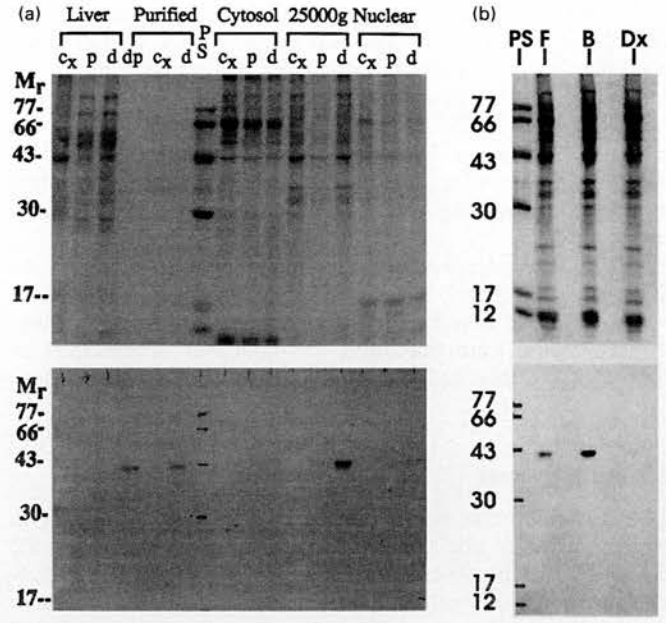

Figure 2 Native photoaffinity labelling of 11β-HSD2

The bottom panels are autoradiographs of labelled protein samples run on the SDS/12.5%-PAGE gels shown in the top panels (stained with Coomassie Blue). $10^{-3} \times$ Apparent M_r of protein standards (PS) are indicated on the left side of the gels. (**a**) Triplicate samples of rat liver microsomes ('Liver'), AMP-chromatography-purified human placental 11β-HSD ('Purified'; similar to fraction 32 in Figure 1) and three human placental subcellular fractions (Cytosol, 25000 ***g*** and Nuclear). All samples were photoaffinity labelled simultaneously with [^{3}H]corticosterone in the presence of 400 μM NAD^+ (lanes d), 400 μM $NADP^+$ (lanes p), both cofactors at 400 μM (lanes dp) or both cofactors plus 50 μM carbenoxolone (lanes c_x). A single protein band at 40000-M_r is labelled in an NAD^+-dependent, carbenoxolone-blockable fashion. (**a**) samples of human placental 25000 ***g*** pellet (mitochondria + heavy microsomes) photoaffinity labelled simultaneously in the presence of 400 μM NAD^+ and [^{3}H]cortisol (F), [^{3}H]corticosterone (B) or [^{3}H]dexamethasone (Dx). Protein loading was < 0.5 μg for 'Purified' lanes; otherwise it was ~ 30 μg/lane.

pellet) was attempted using 0–2.4 M NaCl. The ionic strength clearly affected 11β-HSD activity before centrifugation (activity was highest with 0.3 M NaCl: 160% of that with no NaCl). As $\sim 99\%$ remained insoluble, solubilization was attempted with an extensive screen of detergents. Placental 11β-HSD activity was inactivated by concentrations of detergents required to transform membrane proteins fully into micelles [detergent concentration $\gg$ critical micelle concentration (CMC)]; however, limited though useful solubilization was achieved with gentle conditions (detergent concentration $<$ CMC), being best with zwitterionic detergents with bile acid- or steroid-like head groups. CHAPS (4 mM) in combination with buffer C (which contains 0.3 M NaCl) was found to be optimal, solubilizing 40–45% of the 25000 ***g*** pellet protein and 9.5% of its 11β-HSD activity. On repeated re-extraction of the initially insoluble protein with 4 mM CHAPS, 13–20% of the original 11β-HSD activity could be solubilized. Thus placental 11β-HSD2 appears to be an integral membrane protein that requires factor(s) present in membrane subfractions of the placenta which are not satisfactorily substituted by a wide range of detergents.

Affinity chromatography

Since 11β-HSD uses NAD^+ and glucocorticoids as substrates, columns with ligands that mimic these factors were screened. It was found that one matrix, N-6-linked 5′-AMP–agarose (Sigma; cat. no. A3019), bound substantial 11β-HSD activity that could be eluted with cofactor ($NAD^+ > NADH > 5'$-AMP $\gg$ $NADP^+$). Two other kinds of 5′-AMP–agarose [ribose hydroxyl- (Sigma A8895) and C-8-linked (Sigma A1271)] and two types of NAD^+–agarose were ineffective, with a small percentage (2%) binding to the third type of NAD^+–agarose available (linked at C-8; Sigma N1008), eluting specifically with NAD^+. 11β-HSD activity did not bind to $NADP^+$–agarose, cAMP–agarose or

dexamethasone–agarose (Sigma D4657; 0.5–1.0 μmol/ml dexamethasone immobilized at C-17).

Preliminary studies with this N-6-linked 5′-AMP–agarose revealed it to be very useful for 11β-HSD2 purification. Over a large number of running conditions, a band migrating at approx. M_r 40000 on SDS/PAGE was seen to co-segregate with the 11β-HSD2 activity of the fractions (e.g. Figure 1a). Chromatography for highest purity (e.g. lanes A1 and A2, Figure 1a) suffered from a low yield of active 11β-HSD.

The large-scale preparative conditions adopted (see the Experimental section and Figure 1b) reproducibly gave a 20-fold higher yield (35%) at somewhat decreased, though still 1000-fold, purification (Figure 1a, lanes P1 and P2). The 40000-M_r band (starred) was the most prominent, but contamination persisted at various M_r values, notably several narrow bands very close to (especially above) 40000. These closely flanking bands indicated contaminating proteins that were likely to defeat further purification steps based solely on resolution by size (e.g. gel filtration). Accordingly it was decided to separate by size and charge using 2-D electrophoresis. As this would inevitably result in inactivation of 11β-HSD2 activity, an alternative method of detecting the enzyme was required. This was provided by a novel affinity labelling method.

Affinity labelling

Kinetic studies of 11β-HSD2 purified by AMP–agarose chromatography revealed a very low K_m for corticosterone (14±1 nM) [27], and therefore affinity labelling with this steroid was attempted. As Figure 2 shows, a protein with all the expected characteristics of 11β-HSD was uniquely photoaffinity labelled. This protein was M_r 40000 in size, and was present in the subcellular fractions along with 11β-HSD activity [25000 ***g*** pellet 750 ***g*** pellet (nuclear/debris) ≈ 110000 ***g*** pellet (light microsomes; not shown)], but absent from cytosol. The labelling was NAD^+-dependent, independent of $NADP^+$ and blocked by carbenoxolone. Labelling of fractions after AMP–agarose chromatography showed the labelled protein exactly corresponded to 11β-HSD activity, being present when 11β-HSD was eluted with NAD^+ (e.g. Figure 1b, fractions 87–120) and in the flow-through (fractions 1–30), but absent from the inactive wash fractions. Moreover, the same protein could be affinity labelled with cortisol and dexamethasone (corticosterone ≫ cortisol ≫ dexamethasone; Figure 2b), but there was no labelling with aldosterone. This novel technique appears to result in completely selective labelling of placental 11β-HSD by glucocorticoids, with a potency which parallels their affinities as substrates for the enzyme. Using this technique it was possible to track the 11β-HSD2 protein after activity had been lost, so allowing final purification by 2-D electrophoresis.

The affinity labelling technique developed here is unusually straightforward and specific for 11β-HSD2. Photoaffinity labelling is more commonly described for receptors and binding proteins, and a few such procedures report labelling by unmodified steroid compounds acting as the ligand (e.g. GR by dexamethasone), but even in such cases these 'common' ligands lack the desired specificity and are replaced by compounds developed to allow high specificity (e.g. RU26988 and RU28362 for exchange assays/photoaffinity labelling specific for the GR [42,43]). Affinity labelling of enzymes is much less common by photoactivation, and is more often achieved using a specifically designed activatable compound containing a reactive functional group (usually electrophilic, e.g. bromoacetate) which is attacked by an activating substituent (usually a nucleophilic side chain of an amino acid) in the enzyme's active site {e.g. 3-methoxyoestriol 16-(bromo[2-^{14}C]acetate) labelling of homogeneous placental 17β-HSD type 1 at two histidines in its active site [44]}. Less commonly, enzymes can be photoaffinity labelled by precisely chosen synthetic compounds which, when photoactivated, develop short-lived, highly reactive substituents which label the target enzyme if a suitable amino acid residue is nearby (e.g. UV-induced labelling by 19-nortestosterone acetate of purified Δ^5-3-ketosteroid isomerase from *Pseudomonas testosteroni* [45]). What makes the labelling technique described here particularly unusual is that it can use a physiological substrate in a crude tissue extract containing thousands of different proteins (in contrast to the two examples given above, which used pure enzyme), and labels the enzyme for that substrate with such specificity that analysis reveals only a single spot on 2-D electrophoresis. To our knowledge, the only other study describing photoaffinity labelling of a steroid-metabolizing enzyme by what may be a natural substrate is the labelling of Pseudomonal Δ^5-3-ketosteroid isomerase with testosterone [45]. This, however, is a less avid label than synthetic 19-nortestosterone acetate. Though the parallels are intriguing, that study [45] utilized pure enzyme and ~ 500-fold higher steroid concentrations than were employed here for 11β-HSD2 labelling.

Several findings suggest that the affinity labelling procedure described above occurs within the steroid binding pocket of the active site. Firstly, the affinity labelling is NAD^+-dependent, as is 11β-HSD2 activity. There are a few reports of cofactor-dependent affinity labelling of other dehydrogenases. NAD^+-dependent lactate dehydrogenase is affinity labelled, in the presence of NAD^+, by bromopyruvate on the major catalytic residue (histidine-195) [46]. Secondly, carbenoxolone, an 11β-HSD2 inhibitor, blocks labelling, as does a 1000-fold excess of unlabelled corticosterone. Thirdly, the rank order of potency for glucocorticoid labelling correlates with the substrate affinities of human placental 11β-HSD2.

Finally, the glucocorticoid groups most likely to participate in covalent photoaffinity labelling of 11β-HSD2 are the carbonyl group at C-3 and the C-11 position itself. Labelling via the C-20 carbonyl appears less likely as it is expected to be less photoexcitable, and covalent attachment of the C-20–C-21 grouping (following the common C-17–C-20 cleavage) would not attach any radioactivity, as the steroids are tritiated at C-1, -2, -6 and -7 only. The C-3 carbonyl would be highly photoactivated to produce both alkoxy radicals and reactive ketenes; both of these would react relatively indiscriminately [47], potentially with almost any closely approximated amino acid, but especially with reactive cysteine, lysine, histidine or tyrosine residues. The photoreactive state of C-11 is harder to anticipate, but is likely to be greatly heightened in the active site where it will make the transition to a C-11 carbonyl, a photoexcitable group in its own right.

2-D electrophoresis

Fractions containing enzyme eluted from AMP–agarose were affinity labelled with [^{3}H]corticosterone and then subjected to conventional 2-D electrophoresis (IEF plus SDS/PAGE). After prolonged exposure, gel autoradiography remained negative. Accordingly, NEPHGE gels were run. A strong signal, indicating the presence of a single, affinity-labelled, protein spot of basic pI (~ 9.1) and apparent M_r 40000, was seen on autoradiography (Figures 3a and 3b). It was notable that this was accompanied by other proteins close to 40000-M_r which were not affinity labelled. NEPHGE conditions were optimized (Figure 3) to allow preparative-scale gels with substantially increased yield and reduced smearing of the affinity-labelled protein, which was

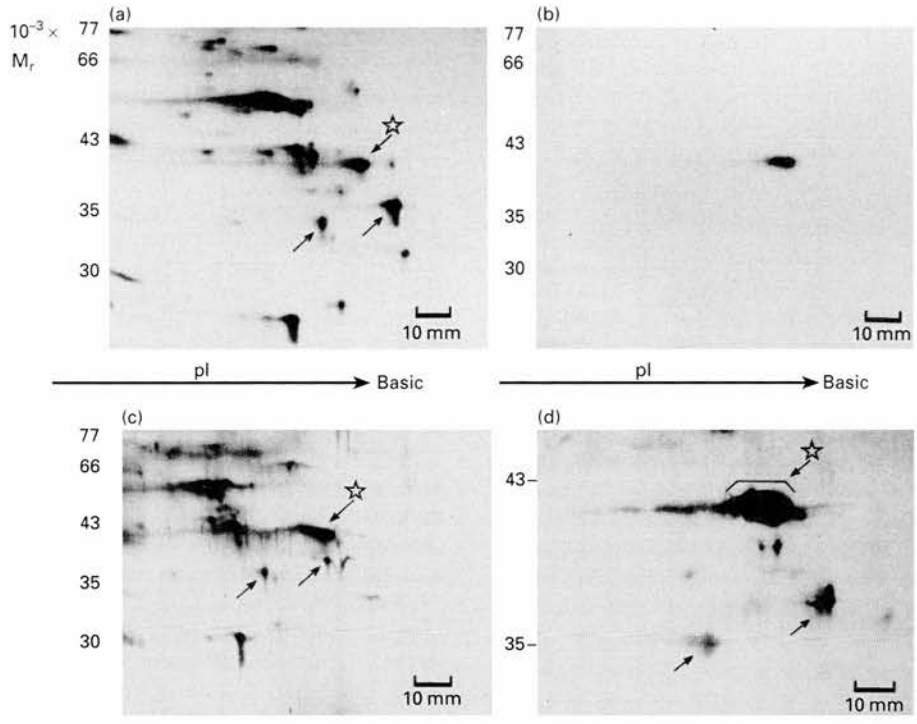

Figure 3 Identification and preparative 2-D electrophoresis of homogeneous 11β-HSD2

Panels (**a**), (**c**) and (**d**) show silver-stained second-dimension gels of NEPHGE 2-D electrophoresis (running to basic pH as indicated). 11β-HSD2 (starred arrow) and two protein contaminants (upward-pointing arrows) are indicated to assist orientation. Panel (**b**) shows an autoradiograph of a gel run in parallel, identical to the one in (**a**), but stained with Coomassie Blue and processed for autoradiography. (**a**) and (**b**) NEPHGE analysis, with an even pH gradient (3–10; see the Experimental section) showing that the protein band(s) at 40000-M_r resolve into multiple protein spots, of which 11β-HSD2 (clearly identified by autoradiography; **b**) is one of the most abundant and basic in pI. (**c**) NEPHGE analysis with the pH gradient expanded at pH 6.5–8.0 to allow better horizontal separation of 11β-HSD2 from nearby proteins at M_r 40000–43000. (**d**) Optimized preparative-scale 2-D electrophoresis for 11β-HSD2 with dodecyl-maltoside-based buffer (greatly reducing streaking) and running the second dimension further to increase the vertical resolution. Note that the 10 mm scales refer to actual gels.

otherwise a major problem. The optimized procedure allowed preparation of over 40 μg of homogeneous 11β-HSD2 protein, which was blotted on to PVDF (Problott) membranes.

Several findings suggest that the 11β-HSD2 membrane environment has polar aspects. Thus moderate ionic strength stimulates activity, the most hydrophobic lipid-like detergents (e.g. Tween 20) are not the most useful for preserving 11β-HSD2 activity, and the 11β-HSD2 protein has a very high pI (> 9). The very basic pI (9.1) is surprising. Proteins this basic are unusual, often being ribosomal or associated with nucleic acid (chromatin or RNA). Rarely are they intrinsic membrane proteins, and this combination suggests interactions of 11β-HSD2 beyond NAD^+ and steroids, perhaps with other membrane proteins or charged lipid. Thus it may well be that interactions of this nature (especially with phospholipid head groups) may be involved in stabilizing the enzyme, as occurs with the related NAD^+-dependent short-chain alcohol dehydrogenase (SCAD) enzyme 3-hydroxybutyrate dehydrogenase [48]. We have not investigated this further, as development of the affinity labelling technique allowed purification of 11β-HSD2 to homogeneity, when activity was not maintained.

Amino acid sequence

PVDF blots were stained with Amido Black and the 40000-M_r target protein accurately excised. Sequencing of PVDF pieces revealed that homogeneous 11β-HSD2 was N-terminally blocked. This is unlikely to be an artifact of the purification as, on sequencing, an amino acid signal was completely absent (rather than just low), and we have obtained high-yield N-terminal sequence from other proteins blotted from gels in the same fashion. *In situ* tryptic digestion of approx. 30 μg of 11β-HSD2 protein produced multiple peptides and several of these were sequenced, yielding in total over 100 residues of amino acid sequence (Figure 4 and Table 1). Peptide G was blocked. This was sub-digested with *S. aureus* V8 protease, yielding a sequencable daughter peptide.

All peptide sequence obtained was unique, confirming 11β-HSD2 as a novel protein. This is the first directly determined 11β-HSD2 peptide sequence from any source. Direct amino acid sequencing provides information about amino acid order and post-translational modification, and avoids some pitfalls of cDNA-predicted amino acid sequencing, such as the use of rare codons (e.g. selenocysteine). There is one potential N-linked glycosylation site (*N*LS; peptide B) which in the native protein, at least in human placenta, appears to be largely free of glycosylation, as the amino acid sequence yield did not drop across this asparagine residue. Peptide C contains a classical SCAD motif (boxed in Table 1) indicating that 11β-HSD2 belongs to this enzyme superfamily. There was one clear 'blank' cycle in the middle of peptide C (cycle 15); it is possible that a modified amino acid naturally occurs here. Further discussion of the sequence is presented in the accompanying paper [30].

Overall purification procedure

Table 2 summarizes the steps in the purification procedure described that allowed isolation of homogeneous 11β-HSD2

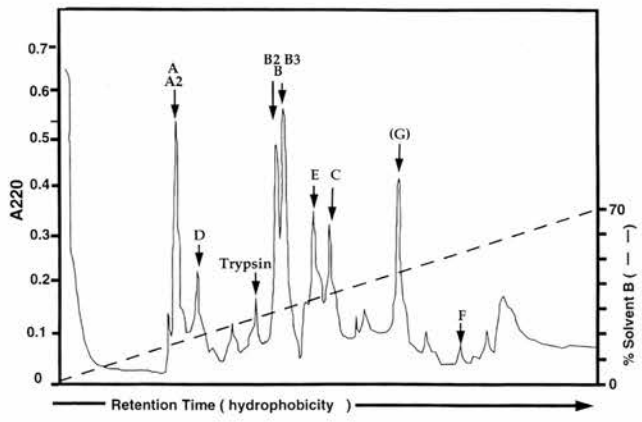

Figure 4 Microbore HPLC profile of 11β-HSD2 tryptic digest

The solid line indicates the elution of peptide peaks (detected by A_{220}; highest peaks corresponding to ~ 500 pmol). The elution positions of trypsin and peaks from which the peptide sequence was obtained (G obtained by V8 sub-digest) are indicated. The digest was loaded on to the HPLC RP300 column equilibrated in solvent A (0.1% TFA in water) and developed with a linear gradient (broken line) of 0–100% solvent B (0.08% TFA and 80% acetonitrile in water) over 45 min at 200 μl/min.

protein from human placenta (> 16000-fold purification) and direct determination of over 100 residues of its internal amino acid sequence. In an accompanying paper [30], the amino acid sequence derived has been used to clone a full-length 11β-HSD2 cDNA from a human placental library which, on transfection into mammalian cells, expresses 11β-HSD2 activity and an approx. 40000-M_r protein which can be affinity labelled by the method described above.

11β-HSD2 substrate binding and reaction mechanism

The primary objective of this work was to accomplish 11β-HSD2 purification. We feel that it also allows conclusions to be drawn which may shed some light on the form of the enzyme's active site and kinetic mechanism. The striking specificity of the photoaffinity labelling of 11β-HSD2 by corticosterone and cortisol, in the presence of NAD^+, demonstrates that 11β-HSD2 exhibits an unusually high, receptor-like, affinity for these glucocorticoids, which may come close to that of GR or MR. This finding underlies the physiological role of 11β-HSD2 in binding, and inactivating, glucocorticoids with high avidity before or in competition with GR and MR, so regulating glucocorticoid access to receptors. 11β-HSD2 is probably not the same as the ~ 45000-M_r 'low-affinity' (K_d ~ 100 nM) dexamethasone binding proteins identified in studies on male rat liver [49], as it differs in localization, hierarchical order of binding affinities and possibly Mg^{2+}-dependence of binding (11β-HSD2 labelling occurs in EDTA-containing buffers). However, 11β-HSD2 does resemble another, incompletely characterized, receptor/binding protein for glucocorticoids: the type 3 corticosteroid (corticosterone) receptor [50,51]. Thus the rank order of affinities determined in binding studies of these 'type 3 sites'

Table 1 11b-HSD2 peptide sequence

The amino acid and yield (in pmol) obtained on sequencing 11b-HSD2 peptides A–G are given by cycle number. Peptide B was sequenced twice (using a second sample from tryptic digestion of purified 11b-HSD2). Cycle C(15) produced no residue signal. Cycles F(5), F(8), F(13) and C(6) produced two residues with similar yield, making assignment difficult. Cycle A(5) is definitely W, but the yield imprecise. Note that the boxed sequence in peptide C is a classical SCAD motif, indicating the likely active site.

CYCLE.	SEQUENCED PEPTIDES [Residues, Yield(pmol)].																			
	A		A2		B			B2		B3		C		D		E		F		G	
1	N	445	L	190	A	180	412	L	315	V	126	I	55	V	411	?	?	?	?	V	45
2	V	426	P	163	L	96	405	L	330	S	119	V	36	L	364	L	406	L	16	N	29
3	G	383	V	182	Q	83	354	Q	318	I	117	T	16	E	231	L	307	D	9	F	27
4	Q	311	A	180	P	76	339	M	316	I	117	V	19	F	333	Q	238	S	7.5	F	15
5	W	>60	T	112	G	66	281	D	170	Q	85	G	15	T	74	L	214	ML	?	G	10
6	E	195	R	31	Q	57	271	L	243	P	46	SI	?	K	37	L	221	G	5	A	9
7	K	97			P	71	243	T	134	G	40	P	15			R	21	F	6	L	7.5
8					G	62	197	K	135	C	40	A	16					TD	?	E	2.5
9					T	39	135	P	170	F	41	G	14					V	8		
10					T	40	116	G	175	K	8	D	12					L	5.3		
11					P	40	121	D	120			M	13					A	3.8		
12					P	33	92	I	167			P	11					T	3		
13					Q	30	82	S	60			Y	10					VP	?		
14					D	18	43	R	28			P	10								
15					A	23	62					-	0								
16					A	25	71					L	8.4								
17					Q	15	43					G	7.4								
18					D	11	16					A	8.1								
19					P	10	21					Y	3.5								
20					N	10	17					G	2.5								
21					L		13					T	1.8								
22					S		6					S	2								
23					P		9					K	1.5								
24					G		9														
25					P		7														
26					S		3														
27					P		3														

Table 2 Summary of purification of 11β-HSD2

Purification using 10 placentas yielded ~ 43 μg of ~ 16000-fold-purified 11β-HSD2. Affinity labelling of tracer amounts of 11β-HSD2 after AMP–agarose chromatography allowed a means of assessing yields beyond this stage. 11β-HSD2 peptide recovery (following *in situ* tryptic digestion, elution from PVDF and microbore HPLC) was generally lower with increasing peptide hydrophobicity (see Figure 4). Activity was measured as nmol/min per mg of protein.

Purification step	Protein	Yield (%)		Purification (fold)		Activity (nmol/min per mg)	Recovery of radioactive tracer (%)
		Step	Cumulative	Step	Cumulative		
Homogenate	46.8 g	–	100	–	–	0.025	–
Subcellular fractionation	5.22 g	67	67	6	6	0.150	–
Solubilization	1.85 g	13	8.7	0.367	2.2	0.055	–
AMP–agarose	640 μg	36	3.15	1040	2290	57.3	–
2-D gel	43 μg	47	1.47	> 7	> 16000	–	100
Blot	30 μg	70	(1.05)	–	–	–	70.2
Eluted peptides	~ 520 pmol (~ 21 μg)	70*	(0.75)	–	–	–	49.8
Initial sequencing yield	445 pmol*	85	–	–	–	–	–

* Best yield.

[50,51] is consistent with the order of potency of the four steroids used to affinity label 11β-HSD2 (Figure 2). 'Type 3 sites' are often regarded as cytosolic, which 11β-HSD2 is not. However, the work originally defining them (in rat kidney) largely used supernatants from centrifugation at 30000 ***g*** for 30 min, which is very similar to the 25000 ***g***/40 min fractionation that we have used. Supernatant from such a spin will probably have approx. 95% of protein from the cytosol, but the remaining 5% will be rich in protein from light microsomes and contain abundant 11β-HSD2. Recent work on isolated renal cortical collecting duct cells characterizes a similar binding site in whole cells (where endogenous NAD^+ may facilitate binding), which may be the renal 11β-HSD2 isoform [51]. The characteristics of placental 11β-HSD2 are thus clearly similar to the 'type 3 sites' in these reports. In the accompanying paper [30] this matter is addressed further by examining the affinities of recombinant 11β-HSD2. However, at present it is not settled whether 'type 3 sites' and 11β-HSD2 are the same entity.

The affinity chromatography data provide some clues to the reaction order and nature of the active site of placental 11β-HSD2. It is likely that 11β-HSD2 binding to N-6-5′-AMP–agarose was via an interaction at the cofactor binding site. 5′-AMP is a 'half molecule' of NAD^+ (nicotinamide comprising the remainder), and instances of N-6-5′-AMP affinity matrices interacting with the cofactor site of NAD^+-dependent dehydrogenases are well documented [52]. We have shown the rank order of potency by which free cofactors elute bound human placental 11β-HSD2 activity from N-6-5′-AMP–agarose to be $NAD^+ >$ NADH > 5′-AMP $\gg$ $NADP^+$ (with 10-fold more 5′-AMP than NAD^+ required for elution) [27]. Thus for free cofactor the binding site prefers NAD^+, so appearing like a NAD^+ cofactor site as opposed to a 5′-AMP allosteric site. When the cofactor is on the affinity matrix and not free, however, the rank order changes to N-6-5′-AMP (yield over 35%) > C-8-NAD^+(yield ~ 2%) > N-6-NAD^+ = C-8-5′-AMP = $NADP^+$ (yield = 0). Clearly the attached spacer arms make a considerable difference, sterically hindering NAD^+ from binding more strongly than 5′-AMP (as occurs with free cofactors). This may be a limitation of the particular affinity matrix products used or simply be because the larger size of NAD^+ makes its binding more sensitive to steric hindrance. A further possibility is that NAD^+ may cause a conformational change on binding (this quite frequently occurs with dehydrogenases), whereas 5′-AMP, filling only a half site, does not. Thus steric interference by the new conformation prevents 11β-HSD2 binding to N-6-NAD^+, but some limited binding is then possible with C-8-NAD^+ (which has the spacer attachment rotated > 90° relative to N-6, both coming from the 5′-AMP half of NAD^+). 5′-AMP, causing no such conformational change, binds well when N-6-linked, but not at all when C-8-linked. The 5′-AMP affinity chromatography results suggest that NAD^+ can bind first to 11β-HSD2 (explaining both binding and elution with NAD^+). The NAD^+-dependence of glucocorticoid affinity labelling suggests that glucocorticoid cannot gain full access to the steroid binding pocket unless NAD^+ has bound first. Affinity chromatography with dexamethasone–agarose was unsuccessful under a range of elution conditions. The finding that under similar conditions 11β-HSD2 is bound well by N-6-5′-AMP agarose, but not at all by dexamethasone–agarose (though dexamethasone is a substrate), may possibly also reflect a 'cofactor first' binding order. By analogy with other dehydrogenases (e.g. lactate dehydrogenase [46]), these findings collectively suggest that a compulsorily ordered ternary complex mechanism may operate for 11β-HSD2, with NAD^+ binding first. Further studies of these matters will assist in mapping the active site and in the design of drugs specifically to inhibit, or possibly constitutively activate, 11β-HSD2.

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Cloning and production of antisera to human placental 11β-hydroxysteroid dehydrogenase type 2

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By inactivating potent glucocorticoid hormones (cortisol and corticosterone), 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) plays an important role in the placenta by controlling fetal exposure to maternal glucocorticoids, and in aldosterone target tissues by controlling ligand access to co-localized glucocorticoid and mineralocorticoid receptors. Amino acid sequence from homogeneous human placental 11β-HSD2 was used to isolate a 1897 bp cDNA encoding this enzyme (predicted M_r 44126; predicted pI 9.9). Transfection into mammalian (CHO) cells produces 11β-HSD2 activity which is NAD^+-dependent, is without reductase activity, avidly metabolizes glucocorticoids (K_m values for corticosterone, cortisol and dexamethasone of 12.4 ± 1.5, 43.9 ± 8.5 and 119 ± 15 nM respectively) and is inhibited by glycyrrhetinic acid and carbenoxolone (IC_{50} values 10–20 nM). Rabbit antisera recognizing 11β-HSD2 have been raised to an 11β-HSD2-(370–383)-peptide–carrier conjugate. Recombinant 11β-HSD2, like native human placental 11β-HSD2, is detectable with affinity labelling and anti-11β-HSD2 antisera, and appears to require little post-translational processing for activity. 11β-HSD2 mRNA ($\sim$ 1.9 kb transcript) is expressed in placenta, aldosterone target tissues (kidney, parotid, colon and skin) and pancreas. *In situ* hybridization and immunohistochemistry localize abundant 11β-HSD2 expression to the distal nephron in human adult kidney and to the trophoblast in the placenta. 11β-HSD2 transcripts are expressed in fetal kidney (but not lung, liver or brain) at 21–26 weeks, suggesting that an 11β-HSD2 distribution resembling that in the adult is established by this stage in human development.

INTRODUCTION

Placental 11β-hydroxysteroid dehydrogenase (11β-HSD) metabolizes the potent glucocorticoids cortisol and corticosterone to inert 11-dehydro products (cortisone and 11-dehydrocorticosterone respectively) and thus protects the fetus from the deleterious effects of the higher glucocorticoid levels in the maternal circulation [1,2]. This protective placental enzyme barrier is very efficient so that almost all maternal cortisol is inactivated [3], ensuring that in late gestation the majority of cortisol reaching fetal tissues is derived from the fetal adrenals [4]. A relative deficiency of this enzyme in both rats and humans correlates with low birth weight [5,6]. Moreover, administration of the 11β-HSD inhibitor carbenoxolone to pregnant rats results in offspring with reduced birth weight and hypertension in adulthood (R. S. Lindsay, R. M. Lindsay, C. R. W. Edwards and J. R. Seckl, unpublished work). These effects parallel the well documented human epidemiological association between low birth weight and hypertension in adult life [7].

The 11β-HSD activity in placenta (11β-HSD2) is due to an isoform distinct from the previously characterized bi-directional NADP(H)-dependent enzyme (11β-HSD1) originally purified from liver [8]. Thus placental 11β-HSD2 has a higher affinity for glucocorticoid, is NAD^+-dependent and acts as an exclusive 11β-dehydrogenase (always inactivating glucocorticoids) [9]. Closely related or identical 11β-HSD2 enzyme activities have been described in rabbit renal cortical collecting duct cells [10], renal tissue from other species [11], and several human fetal tissues [12]. This enzyme activity in the distal nephron excludes glucocorticoids from otherwise non-selective mineralocorticoid receptors, so producing aldosterone selectivity in the face of a 1000-fold molar excess of circulating cortisol [13,14]. In the accompanying paper [15] we report the purification to homogeneity of human placental 11β-HSD2. Here we describe the isolation of a cDNA clone encoding human placental 11β-HSD2, the tissue distribution of the corresponding mRNA and studies examining the characteristics of the expressed protein and enzyme activity. In addition, we have raised antisera allowing the detailed localization of the 11β-HSD2 protein.

EXPERIMENTAL

Materials

^3H-, ^{35}S- and ^{32}P-labelled compounds, Hybond N and Hybond enhanced chemiluminescence (ECL) membranes, Hyperfilm $B_{max.}$, Sequenase v2 and the ECL Western blotting systems were purchased from Amersham International (Little Chalfont, Bucks., U.K.). *Asc*I was obtained from New England Biolabs (Hitchin, U.K.). PGEM-11zf(+) plasmids, micrococcal nuclease-treated canine pancreatic microsomes (cat. no. Y4041) and other restriction enzymes were obtained from Promega (Southampton, U.K.). Synthetic oligonucleotides were synthesized by Oswel DNA Service (Edinburgh, Scotland, U.K.). DNA size markers (1 kb ladder), Lipofectin, media and reagents for tissue culture were purchased from Gibco–BRL (Paisley, Scotland, U.K.).

Abbreviations used: 11β-HSD(2), 11β-hydroxysteroid dehydrogenase (type 2); SAME, syndrome of apparent mineralocorticoid excess; ECL, enhanced chemiluminescence; RT-PCR, reverse transcription-PCR; UTR, untranslated region; TBS, Tris-buffered saline; DHEA(S), dehydroepiandrosterone sulphate; SCAD, short-chain alcohol dehydrogenase.

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The nucleotide sequence data reported have been submitted to the Genbank/EMBL/DDBJ Nucleotide Sequence Databases under accession no. U26726.

Vectastain *Elite* ABC and DAB reagent system for immunohistochemistry was purchased from Vector Laboratories (Peterborough, U.K.). Reagents and molecular biology grade chemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Degenerate primer PCR and cloning of 11β-HSD2 fragment

Tissue was snap-frozen and RNA was extracted as described [16]. RNA was treated with DNase I, re-extracted to remove any contaminating genomic DNA and reverse transcribed using the Promega Reverse Transcription system (cat. no. A3500) according to the manufacturer's instructions. Inosine-containing degenerate primers were designed based on the amino acid sequence of five 11β-HSD2 tryptic peptides: A, B, B2, C and D (t = top strand, b = bottom strand) [15]. The primers from peptides B and C generated the most useful results and had the following sequences (where I is inosine): Bt, 5′-CA(A/G)GA(C/T)GCIGCICA(A/G)GA(T/C)CCIAA-3′; Bb, 5′-A(A/G)(A/G)TTIGG(A/G)TC(C/T)TGIGCIGC(A/G)TC-3′; Ct, 5′-(A/T)(C/G)ICCIGCIGGI(A/G)(A/C)IATGCCITA-3′; Cb, 5′-AGCICCIA(A/G)IIIIGG(A/G)TAIGGCAT-3′. Initial screening used the following protocol. A 5 μl sample of human placental cDNA was heated, under mineral oil, at 96 °C for 10 min and placed on ice. The reaction mixture was then added in a volume of 45 μl to give a final reaction mix containing 70 pmol of each degenerate primer, 50 μmol of dNTP and 2 units of Taq polymerase in 1 × PCR buffer (Promega, Southampton, U.K.). Reactions were placed into a thermal cycler (HB-TR1; Hybaid, Teddington, U.K.) with the block held at 90 °C and the programme commenced: 5 cycles of 60 s at 95 °C, 45 s at 43 °C, 43 → 55 °C at 1 °C/3 s and 90 s at 72 °C, followed by 35 cycles of 60 s at 95 °C, 60 s at 50 °C and 90 s at 72 °C, and finally 10 min at 72 °C. Subsequently a specific DNA product (of 531 bp) was efficiently amplified using the Ct/Bb primer pair and the following programme: five cycles of 60 s at 95 °C, 60 s at 47 °C, 47 → 50 °C at 1 °C/5 s, 50 → 55 °C at 1 °C/2 s and 150 s at 72 °C, followed by 40 cycles of 60 s at 95 °C, 60 s at 50 °C, 50 → 55 °C at 1 °C/s and 150 s at 72 °C, and finally 10 min at 72 °C. This allowed the 531 bp product to be directly cloned into pCRII (TA Cloning system v2; Invitrogen, San Diego, CA, U.S.A.: cat. no. K2000-01) to yield clone pCRIICtBb.

PCR screening of a pcDNA1 human placental cDNA library

Specific PCR primers nested within this 531 bp fragment were designed [top primer (SCt), 5′-ATCCGTGCTTGGGGGCCTA-TGGAACCT-3′; bottom primer (SBb), 5′-CTGCAGTGCTCG-AGGCAGACAGTGACT-3′]. These produced a strong band of the expected size (455 bp) as the only product amplified by reverse transcription (RT)-PCR of human placental RNA. A human placental cDNA library in pcDNA1 (Invitrogen; A900-11) was then screened by increasing dilutions using this 455 bp product to detect positive pools and adapting the PCR screening method of [17] for use with plasmid libraries. Other specific primers were used to cross-check positives. PCR with one primer to the pcDNA1 vector arm and the other to the insert was used to estimate clone insert size. A partial 11β-HSD2 clone was isolated. On re-screening only one clone (> 1.9 kb) long enough to be full length [with coding region (40000-M_r protein) plus 3′ untranslated region (UTR) being ⩾ 1.7 kb] was found, but this produced no 11β-HSD activity on expression in CHO cells.

Screening of a λDR2 cDNA library

A λDR2 human placental cDNA library (Clontech, Palo Alto, CA, U.S.A.; HL1144x), with a higher proportion of longer cDNA inserts, was screened by conventional means using the incomplete 11β-HSD2 sequence isolated from the pcDNA1 library. Briefly, 700000 plaques were plated and duplicate filter lifts were made, denatured, fixed, rinsed, dried and UV cross-linked before hybridization in SSC/formamide buffer (6 × SSC, 50 % formamide, 5 × Denhardt's solution, 0.5 % SDS, 100 μg/ml salmon sperm DNA) with random primed [^{32}P]dCTP-labelled probe from the incomplete 11β-HSD2 sequence. Washes were SSC/SDS-based, finishing with one 15 min wash at 65 °C in 0.2 × SSC/0.1 %SDS. Positives from the primary screen were purified by a secondary screen at low density. Secondary positives were converted from phage (λDR2) to plasmid (pDR2) clones by means of the endogenous CRE/LOX recombinase of the host bacterial strain (*Escherichia coli* AM1) [18] and were tested for 11β-HSD2 enzyme activity by transfection into CHO cells, as described below. Nucleotide sequences were determined following sequencing of both strands by the dideoxy termination method using Sequenase v2 (Amersham/USB).

Nucleic acid and protein sequence analysis

The 11β-HSD2 cDNA sequence and predicted 11β-HSD2 protein sequence were analysed using the computing facilities at the HGMP Resource Centre (Cambridge, U.K.) [19], specifically PredictProtein [20] and the GCG package [21]. Protein secondary structure was predicted by three methods, according to (1) Chou–Fasman as amended for overall structure probability [22,23], (2) Garnier–Osguthorpe–Robson [24], (3) and Rost–Sander [20]. The features described are for regions where there was no disagreement in predictions, while Figure 2 (middle panel) also extends illustration to areas (grey regions) with complete concordance between two predictions and where the third 'dissenting' prediction is not in complete agreement. Assessment of hydrophobicity was based on the Kyte–Doolittle index [25], while prediction of flexible and exposed/buried regions was according to the methods of Karplus–Schulz [26] and Emini et al. [27] respectively. Estimates of percentage identity in nucleic acid and protein alignments used the GAP algorithm [21] with standard settings of gap weight = 3 and length weight = 0.1 for protein alignment.

In vitro translation

The 11β-HSD2 cDNA was subcloned into pGEM-11zf oriented so that the 5′ end of the cDNA was adjacent to the vector T7 promoter. *In vitro* translation was performed with a T7 polymerase driven, rabbit reticulocyte-based, coupled transcription/translation system (TNT lysates; Promega; L4610), to which a methionine-deficient amino acid mixture and [^{35}S]methionine were added. In a standard volume of 25 μl, 0.5 μg of subcloned 11β-HSD2 plasmid DNA was added. Microsomal co-translational processing was examined by including 0–4.5 μl of micrococcal nuclease-treated canine pancreatic microsomes in the standard 25 μl incubation at 30 °C for 90 min. Control reactions to verify signal peptide cleavage (0.1 μg of *E. coli* β-lactamase mRNA) and glycosylation (0.1 μg of *Saccharomyces cerevisiae* α-factor mRNA) activities of the microsomes were carried out in parallel. For autoradiography, 0.05–5 μl of each reaction was run per lane on SDS/PAGE, stained with Coomassie Blue, processed in Entensify fluoroautoradiography solutions (NEN/DuPont, Stevenage, U.K.), dried and exposed to Kodak X-OMAT AR film.

CHO cell transfection

CHO cells were maintained in Dulbecco's modified Eagle's

medium supplemented with 15% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 200 mM glutamine. At 24 h prior to transfection cells were seeded on to dishes at a density of 2×10^6 cells/100 mm plate. For transfection, 5 μg of DNA (in 800 μl of Optimem) was mixed with 42 μl of Lipofectin (in 800 μl of Optimem) and incubated for 15 min at room temperature. Optimem was added to 10 ml and the mixture was gently overlaid on to cells which had been washed in Optimem. After 24 h the Optimem/DNA mix was removed and replaced with normal medium. Cells were harvested 24 h later. The human placental 11β-HSD2 cDNA was transfected into cells using the clone (in pDR2) isolated from library screening and there were appropriate controls for transfection efficiency. Assays of 11β-HSD activity were either with intact cells ([^{3}H]steroid added to the medium within the last 24 h, as described [28]) or using homogenates of scraped cells (as below).

Kinetic and inhibitor studies

CHO cells were scraped, homogenized [2×10^6 cells/0.5 ml of Buffer C (0.02 M Tris, pH 7.7, 10% glycerol, 1 mM EDTA, 300 mM NaCl)], centrifuged briefly (15000 *g*, 15 s to pellet heavy debris, and the supernatant assayed for protein concentration and 11β-HSD2 activity essentially as described [15]. 11β-HSD2 assays contained 400 μM NAD^+, unless otherwise stated, and were analysed by HPLC when the [^{3}H]steroid concentration was > 2.5 nM; TLC-based analysis was also used at [^{3}H]steroid concentrations < 5 nM. Reaction products were identified (HPLC and TLC) by comparison with steroid standards run in parallel. Incubations were for 60 min (120 min for dexamethasone). Kinetic parameters were calculated from the initial-velocity determinations, obtained with experiments performed with a wide range of substrate concentrations (0.3, 0.4, 0.8, 1.5, 2, 3, 4, 8, 15, 20, 40, 80 and 150 nM [^{3}H]steroid, with 80 and 150 nM omitted for corticosterone). Enzyme concentrations giving less than 30% conversion were used. Control ('vector-only' transfected cells) and blank assays were carried out in parallel.

11β-HSD2 photoaffinity labeliing

This was carried out as described in the accompanying paper [15], with the labelling performed at 0 °C. Transfected cells were homogenized, briefly centrifuged (1000 rev./min × 15 s) to pellet lumpy debris, and the supernatant labelled at 0.25 mg of protein/ml. Labelling reactions of the placental 25000 *g* pellet (at ~ 0.15 mg of protein/ml) were run in parallel. The post-labelling samples were acetone-precipitated and analysed by electrophoresis (SDS/PAGE) and fluoroautoradiography.

Northern hybridization

Adult human tissue samples were obtained at surgery, frozen within 10 min and stored at −80 °C. Most samples were normal tissue removed adjacent to a tumour on resection; this was the case for kidney (with adjacent adrenal), parotid, colon (splenic flexure), breast (with adjacent skin and dermis) and stomach (with distal oesophagus). Normal ovary (pre-menopausal) was obtained at hysterectomy. Pancreas was an unaffected area in a pancreatic tail resected for chronic pancreatitis. Liver was from a partial hepatectomy in a young woman to remove a hepatic cyst (benign). Placenta was from a normal delivery. Regions of a normal human brain obtained *post mortem* (36 h) were also dissected. RNA was extracted as described [16], separated on denaturing agarose/formaldehyde gels (~ 10 μg/lane) and blotted on to Hybond N membrane (Amersham). A human multiple tissue Northern blot was purchased from Clontech (7756-1). Highly purified poly(A) RNA (2 μg) from fetal tissues [brain, lung, liver (female) and kidney] recovered following spontaneous abortions (at least two specimens pooled for each organ) were run in each lane and blotted. The exact age ranges were: fetal brain, 21–26 weeks; fetal lung, 22–23 weeks; fetal liver, 22–26 weeks; fetal kidney, 19–23 weeks. Blots were hybridized with a randomly primed ^{32}P-labelled p11β2 *Asc*I–*Dra*I fragment (bases 217–1737; see Figure 2) at 55 °C overnight in hybridization buffer (0.2 M sodium dihydrogen phosphate, 0.6 M disodium hydrogen phosphate, 5 mM EDTA, 6% SDS and 100 μg/ml denatured herring testis DNA). Washes were SDS/SSC-based, finishing with 0.1 × SSC/0.1% SDS at 65 °C followed by autoradiography (−70 °C; 3–8 days).

In situ hybridization

Cryostat sections (10 μm) were cut from frozen samples of human kidney (normal tissue from the opposite pole to a discrete renal cell carcinoma) obtained at surgery and normal placenta. Sections were mounted on to gelatin- and poly(L-lysine)-coated slides, and stored at −80 °C. Slides were post-fixed in 4% paraformaldehyde, washed in 2 × SSC and incubated with pre-hybridization buffer for 3 h at 50 °C, as previously described [29], before hybridization with SP6-transcribed [^{35}S]UTP-labelled anti-sense cRNA probes from *Xba*I-linearized pCRIICtBb (531 bp of p11β2; bases 654–1184; dashed box in Figure 2, top panel). Sense controls used T7-transcribed cRNA primed from *Hin*dIII-linearized pCRIICtBb. RNA probes were denatured, added at a final concentration of 10×10^6 c.p.m./ml in hybridization buffer, applied to slides as described [29] and incubated overnight at 50 °C. Following hybridization, sections were rinsed twice in 2 × SSC, treated with RNase A (30 μg/ml, 60 min, 37 °C), and washed to a maximum stringency of 0.1 × SSC at 60 °C for 60 min. After dehydration in increasing concentrations of ethanol, slides were exposed to autoradiographic film (Hyperfilm $B_{max.}$). Slides were dipped in photographic emulsion (NTB-2; Kodak) and exposed in a light-tight box (D19; Ilford) for 5 weeks, before being developed and counterstained with 1% pyronin.

Raising of antisera and immunohistochemistry

Solid-phase synthesis of an 11β-HSD2 peptide HCLPRALQPGQPGT (residues 370–383; see Figure 2) with high predicted antigenicity was carried out. The peptide was N-terminally coupled to keyhole limpet haemocyanin and rabbits were inoculated with the conjugate in Freund's adjuvant, boosting monthly. Antisera highly specific for 11β-HSD2 and reacting to the peptide were obtained after the fourth boost. Western blots were performed with the 11β-HSD2 antisera, at 1:10000 dilution (as primary antibody), using the Amersham ECL system and methods recommended by the manufacturer.

Immunohistochemistry including pre-absorption with excess 11β-HSD2-(370–383)-peptide antigen

Sections of human tissue, stored at −80 °C, were thawed into neutral formalin fixative (for 8 h), paraffin-processed and 4 μm sections cut. After drying, sections were dewaxed, hydrated and treated in 3% H_2O_2 (20 min) and blocked with 20% sheep serum in TBS (Tris-buffered saline, pH 7.6, with 1% BSA and 0.1% sodium azide). This diluent was also used for primary and secondary antibodies. Immunostaining, using an avidin–biotin

complex (ABC) method at room temperature, was as follows: primary antibody (1:2000), 30 min; TBS wash; secondary antibody [1:400 biotinylated sheep anti-rabbit $F(Ab)_2$ fragments; Boehringer Mannheim, Lewes, W. Sussex, U.K.], 30 min; TBS wash; Vectastain ABC *Elite* reagent, 30 min; diaminobenzidine substrate/chromagen reagent, 5 min. Sections were counterstained in Mayer's haematoxylin, dehydrated, cleared and mounted. Primary antibody preparations used were (a) 11β-HSD2 antisera, (b) preimmune serum from the same rabbit and (c) 11β-HSD2 antisera pre-absorbed for 16 h with 11β-HSD2-(370–383)-peptide at 50 μg per ml of diluted antibody. Both (b) and (c) were used as controls for (a).

RESULTS AND DISCUSSION

Cloning of 11β-HSD2 from human placenta

Degenerate primers (based on five 11β-HSD2 tryptic peptide sequences, both strands) were used in all pairwise combinations in an initial RT-PCR screen carried out on human placental mRNA. Combinations involving primer Bb resulted in the amplification of several particularly prominent DNA products, the majority of which appeared to be independent of the top primer used (Figure 1, lanes 1–4), and indeed PCR with Bb alone reproduced many of these bands. A clear exception was the PCR product arrowed in Figure 1 (lane 2); this was specific to the CtBb primer pair and became the major product (Figure 1, lane 5) using more stringent PCR conditions. This CtBb fragment was directly cloned and sequenced, revealing a single open reading frame spanning its length (531 bp) and with a predicted amino acid sequence encompassing four 11β-HSD2 peptides (C, B, A and B3), including a short-chain alcohol dehydrogenase (SCAD) motif (YXXXK; see below). This CtBb fragment was used (a) to design a synthetic peptide to raise antisera (see below) and (b) to isolate a complete 11β-HSD2 cDNA.

A human placental cDNA library in pcDNA1 was initially screened by a PCR-based approach and a 1177 bp partial clone

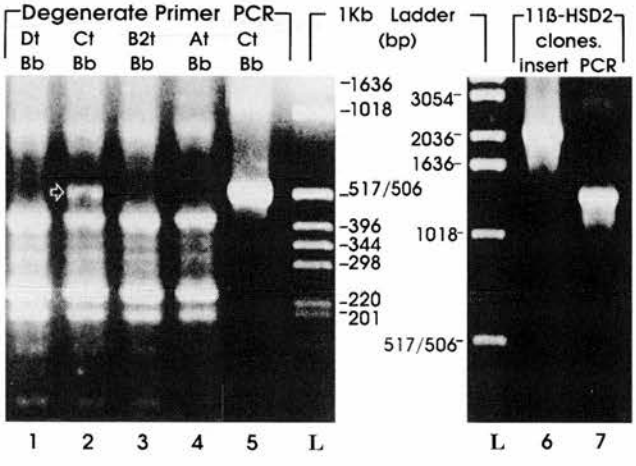


Figure 1 PCR to amplify 11β-HSD2 sequences

Ethidium bromide-stained agarose gels showing analysis of PCR amplifications. Lanes 1–5, RT-PCR on human placental mRNA using degenerate primers based on 11β-HSD2 peptide sequences A–D. Primers used (Xt = top; Xb = bottom; X = peptide) are indicated above the corresponding lane. Lanes 1–4, initial PCR conditions used; lane 5, more stringent amplification used, yielding only the CtBb specific product (531 bp) identified initially (arrowed, lane 2). Lanes 6 and 7, PCR (using vector-specific primers flanking the cloning sites) of 11β-HSD2 inserts of a 1179 bp partial pcDNA1 library clone [1157 bp + 22 bp poly(A); lane 7] and the 1919 bp [1897 bp + 22 bp poly(A)] pDR2 clone containing the full coding region (lane 6). Lanes L, 1 kb ladder (Gibco–BRL.)

isolated (Figure 1, lane 7) which contained the CtBb fragment, 45 bp further 5′ and 603 bp further 3′ [reaching to the 3′ end of the cDNA with a 22 bp poly(A) tail]. Conventional screening of 700000 plaques from a human placental DR2 library (Clontech), using the incomplete 11β-HSD2 sequence as a probe, allowed isolation of a single clone which, when converted to a pDR2 plasmid form (see the Experimental section), produced very abundant, NAD^+-dependent, 11β-HSD2 activity in transfected CHO cells. This clone was designated p11β2.

Characteristics of the cDNA clone and predicted 11β-HSD2 protein sequence

General features of the 11β-HSD2 cDNA clone

This active 11β-HSD2 clone, p11β2, contained a 1897 bp cDNA with 22 bp poly(A) tail (1919 bp total), and has a G+C-rich composition (63.25%). The 5′ end of the cDNA (first ~385 bases) is extremely G+C-rich (79.2%) and contains a 'CpG island' [30] with 56 occurrences (in 385 bases) of this usually scarce dinucleotide. The G+C and CpG content falls abruptly beyond this region (to 55% G+C; CpG frequency falls 4-fold). These features, which extend to the start of the 11β-HSD2 cDNA, are suggestive of a 5′ transcription regulatory region. The best candidate for initiation of translation is the ATG codon beginning 134 bases from the 5′ end of the cDNA (set to +1 in Figure 2, top panel); this is in a good context for initiation of translation, with 9/10 matches to the putative ideal ribosome binding site (GCCGCCATGG) [31], and is within the longest open reading frame (−63 → +1215; Figure 2), defining a predicted coding region of 1215 bp (+1 → +1215), flanked by a 5′ UTR of 133 bp (−133 → −1) and a long 3′ UTR of 549 bp (+1216 → +1764). The predicted coding region encodes a 405-amino-acid protein (calculated M_r 44126) encompassing all the 11β-HSD2 peptide sequence derived from digests of the purified protein [15] (boxed in Figure 2). The most abundant amino acids are leucine (68 residues in 11β-HSD2), alanine (46) and glycine (32), which are all highly represented in proteins in general, followed by proline (32) and arginine (30). The high arginine content seems responsible for the very basic nature of the 11β-HSD2 protein, so clear during purification, as basic residues (Arg = 30, Lys = 12, His = 7) markedly outnumber acidic ones (Asp = 15, Glu = 15), giving a predicted net positive charge (+19) and a very basic predicted isoelectric point of 9.92 for the 11β-HSD2 polypeptide. Nine cysteine residues are present, one of which occupies the position at which a blank cycle resulted on sequencing of an 11β-HSD2 peptide (cycle 15, peptide C) [15], suggesting that this residue may be particularly reactive or modified in the native protein.

Detailed analysis of the predicted structure of the 11β-HSD2 protein

Analysis of the primary structure (Figure 2, top panel) and the predicted secondary structure (Figure 2, middle panel) of the 11β-HSD2 protein suggests four distinct regions. The most N-terminal region (region I) begins with an exposed loop (Met-1–Gly-9) followed by a very leucine/alanine-rich area (60% of residues 11–73). This has some incomplete repeats (AALALLAAL; residues 36–44; close variants beginning at residues 16 and 56) and appears likely to have secondary structure broken by helix-breaking residues (Gly-31 and Pro-33; Pro-53–Pro-54–Pro-55) into three buried, predicted α-helical, segments centring on residues 16–22, 35–41 and 63–69 respectively. The region has imperfect heptad symmetry, and in such a secondary structure is likely to form a particularly hydrophobic

face along which leucines align, there being two such potential axes: (1) L14-L21-L28-L35-A42, stretching around to the nearby helical face L13-L20-D27-L34-L41, and (2) reaching further C-terminal in the region L23-L30-A37-L44-L51-L58-G65-L72. On the helical aspect opposite to these axes all seven positively charged residues in the area line-up: R18-R25-R32—R74 and R29–R50–R71. Such a structure is suggestive of a domain with a leucine-zipper-like tendency to form protein–protein interactions (often dimerization) along the hydrophobic axes.

The second region (region II), which unlike the rest of the protein has predicted secondary structure rich in β-sheets, contains three motifs characteristic of members of the SCAD superfamily. Firstly, the putative cofactor-binding site motif (V/I)TGXXXGXG is present (ITGCDSGFG; residues 87–95) in an area with secondary structure predicted to incorporate buried β-sheet (residues 84–88), buried loop (89–92) and an exposed helix (96–102). Secondly a 'substrate positioning' motif, noted in previous alignments of SCAD enzymes [32], which is of the form [LFVM][VI][NL][N][AVH(F)][GI], is present (LVNNAG; residues 164–169). The major predicted features of this substrate positioning area are an exposed loop (residues 156–160)/buried β-sheet (161–166+) being preceded by an α-helix (centred on residues 145–152) and exposed flexible region likely to loop around Pro-142–Gly-143. Thirdly, the putative catalytic motif YXXXKX(SAG) is present (YGTSKAA; residues 232–238). This motif, which is predicted to form a small loop around Gly-233, is in the centre of a stretch predicted to have flanking areas consisting of loop → buried β-sheet → loop structures (at 211–223 and 252–264). A long hydrophobic α-helix (residues 236–250) bridges the motif-loop/sheet/loop stretch to the C-terminal side; however, the secondary structure is unclear for the region (residues 224–231) bridging between the motif and the loop/sheet/loop on the N-terminal side. The equivalent of this 'upstream bridging region' in the related SCAD enzyme 3α,20β-HSD was shown to be part of the steroid substrate (cortisone) binding pocket in X-ray crystallographic studies [33].

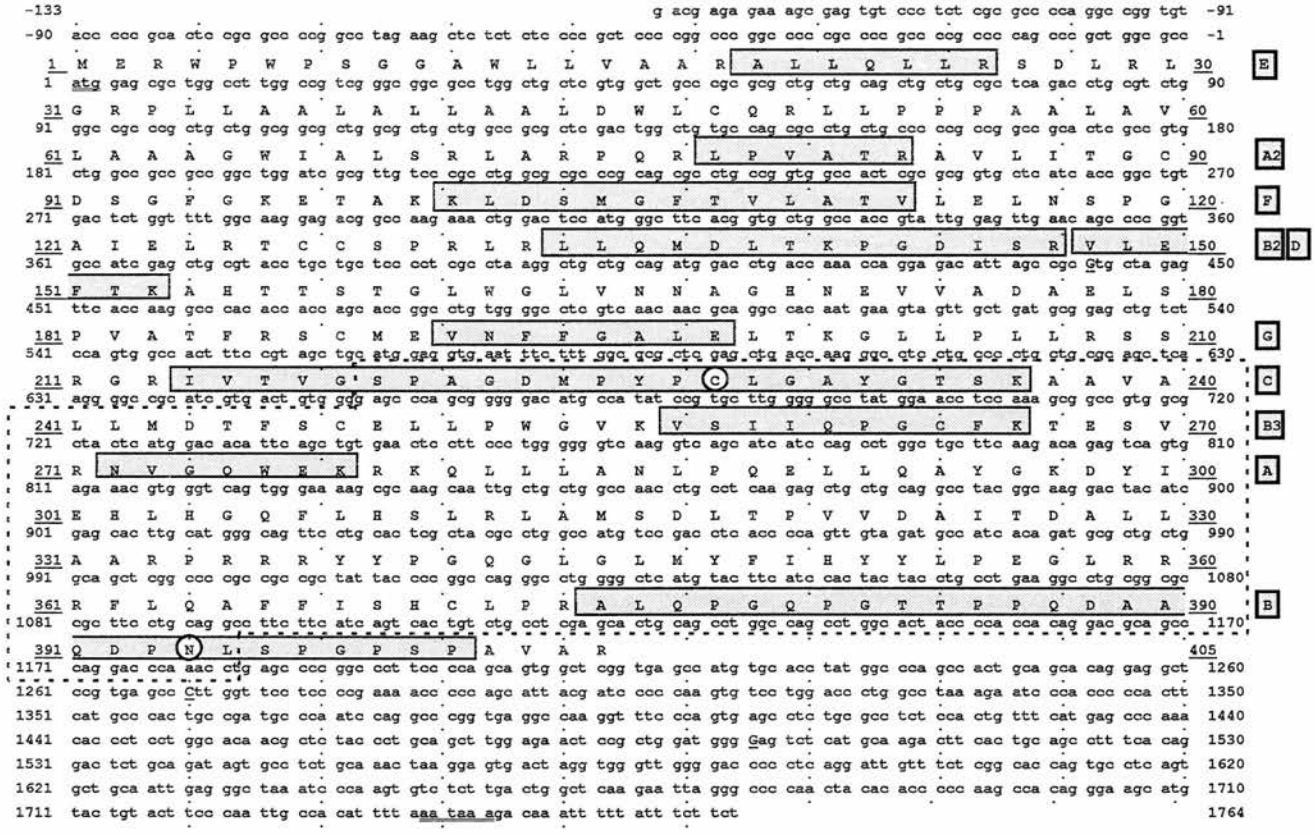
Region III (residues 275–370) has three positively charged segments, EKRKQ, ARPRRRY and LRRRF (residues 277–281, 332–338 and 358–362 respectively). Such segments may form charge interactions with negatively charged molecules or exert electrostatic influence on a substrate. In the context of a membrane protein, interactions with phospholipids helping to stabilize or anchor the protein are particularly likely and often occur adjacent to helical transmembrane domains (e.g. glycophorin [34]). Indeed most of the region between the charged segments has predicted helical secondary structure, especially residues 274–294, 298–312 and 324–331.

Finally region IV, the C-terminal region, is proline/glycine-rich (36% of residues 373–405) and is predicted to form a flexible area with several exposed loops. It contains the only potential N-glycosylation site in 11β-HSD2. The potential for glycosylation at this motif is weakened as it is flanked by prolines. Moreover, amino acid sequencing yields across the B peptide (Figure 2) suggested that Asn-394 was largely unglycosylated in the native protein from human placenta [15].

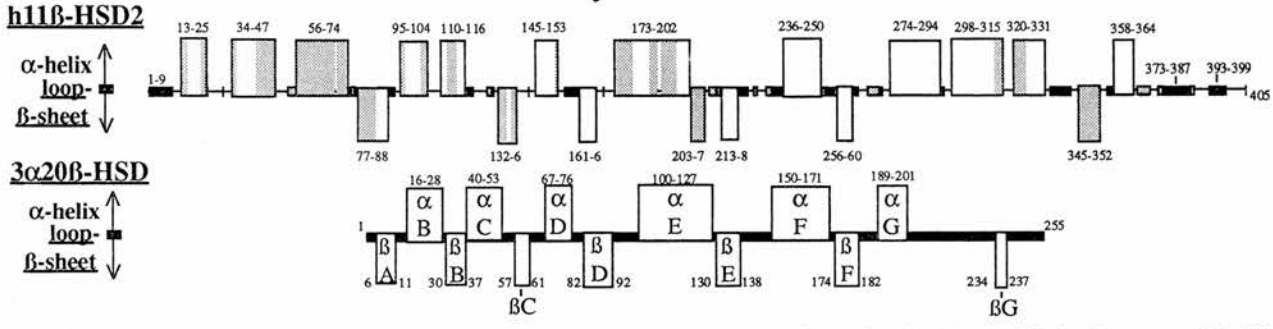
Although much of the secondary structure can be predicted with some certainty, there are important aspects of the higher-order structure of 11β-HSD2 that are unclear. As 11β-HSD2 is an intrinsic membrane protein from a family of enzymes (SCADs) thought usually to be tetrameric in the native form, it probably contains both transmembrane segments and dimerization interfaces. The N-terminal leucine/alanine-rich region (residues 11–73) may potentially fulfil either role, whereas the helical/charge cluster region (residues 275–370) contains predicted hydrophobic helical segments which are good candidates as possible transmembrane regions. Dimerizing elements outside the N-terminal region seem likely, as a number of other SCADs which lack such a region have been demonstrated to form tetramers. Figure 2 (middle panel) also illustrates the secondary structure of the related SCAD enzyme 3α,20β-HSD as determined from X-ray crystallography studies. There is a very striking similarity within region II (the SCAD region), with almost all the major structural features from βA–βF of 3α,20β-HSD being represented clearly in the predicted 11β-HSD2 structure [the only exception being that βB corresponds to a segment of 11β-HSD2 (residues 104–109) with uncertain secondary structure]. Studies on 3α,20β-HSD have identified areas involved in steroid and cofactor binding and in dimerization; some of the corresponding regions in 11β-HSD2 may have similar functions The dimerization interfaces of 3α,20β-HSD include the the region between the LVNNAG and YXXXK SCAD motifs (αE–βE–αF) and the most C-terminal β-sheet element (βG) [33].

Sequence similarities to human placental 11β-HSD2

A search of sequence databases shows that the most clearly related proteins are SCAD members (Figure 2, bottom panel), the closest being microsomal NAD^+-dependent human 17β-HSD type 2 [35] (38.9% amino acid identity), retinal pigment epithelium NAD^+-dependent bovine 11-*cis*-retinol dehydrogenase (35.5% identity) and mitochondrial human NAD^+-dependent enzyme 3-hydroxybutyrate dehydrogenase [36] (32.6% identity). There is 28.3% identity to human 11β-HSD type 1 [37] (microsomal; $NADP^+$-dependent). Similarities of the four 11β-HSD2 regions (I–IV) are shown in Figure 2 for these enzymes and others with some functional similarity that are related to 11β-HSD2. The three SCAD motifs referred to above are highly conserved, although the middle motif is atypical in 11β-HSD1 and is shifted in relative position or absent in 3β-HSD2. This latter enzyme appears to have the other two SCAD motifs in the same orientation and with spacing typical of SCADs. This highlights the fact that members of the bifunctional 3β-HSD family (which share high sequence identity and also have ketosteroid isomerase activity) fit somewhat uncomfortably into the SCAD superfamily, and it is unclear whether they should be considered as SCADs or as a separate family. NAD^+-dependent 11β-HSD enzymes have been expression-cloned from kidney in sheep [38] and human [39]. The sheep kidney 11β-HSD2 has 78.5% identity at the protein level (82.6% at the nucleic acid level). Similarity is non-uniform across regions I–IV at both the protein (Figure 2) and nucleic acid levels; thus sheep 11β-HSD2 has 94.2%, 88%, 80% and 65% identity with human 11β-HSD2 at the cDNA level in regions I–IV respectively. The 5′ UTR and most of the 3′ UTR have ~ 70% identity; however, this rises sharply again between bases 1536 and 1624 (Figure 2, top panel) (91% identity; reason unclear) and for sequences adjacent to the polyadenylation motif aaataa (92%; bases 1731–1764; Figure 2). The human renal clone is similar, but not identical, to the cDNA from placenta reported here. The renal clone lacks the first 25 bases (−133 → −109), has two deletions in the 3′ UTR (bases 1270 and 1495; Figure 2) and a base substitution at 442 resulting in a change in the predicted amino acid sequence of Val-148 → Leu. At the points of difference, the sequence reported above has been confirmed in cDNA that we have sequenced derived from a second placenta, and the Val-148 residue is clearly present in human placental 11β-HSD2 as it is the first amino acid of the D peptide sequence derived from purified 11β-HSD2 tryptic digests. Although it is most likely that these differences arise from polymorphisms, there may be isoform

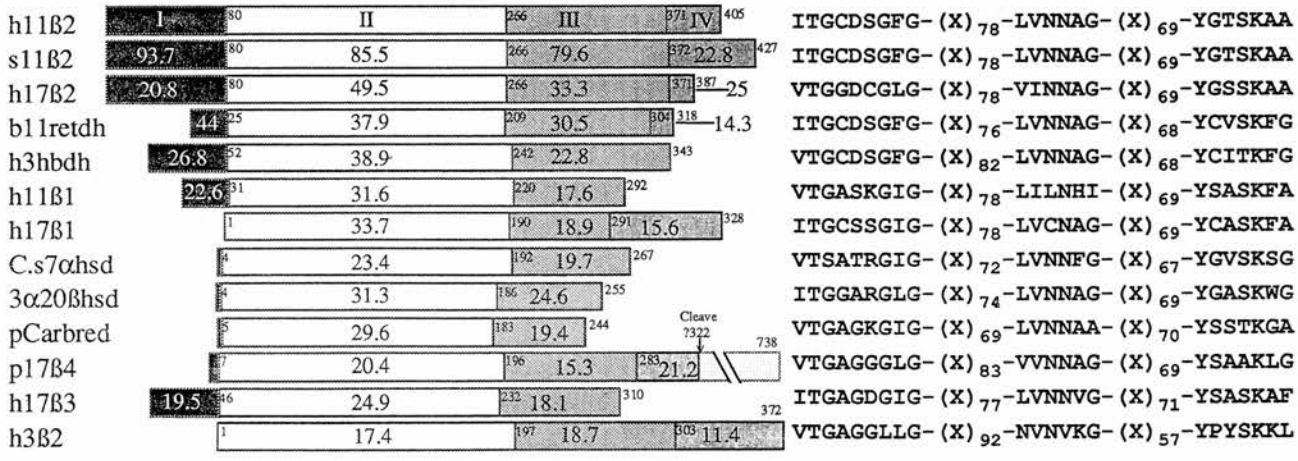


Predicted Secondary Structure.



Percent Amino Acid Identity

Short Chain Alcohol Dehydrogenase Motifs



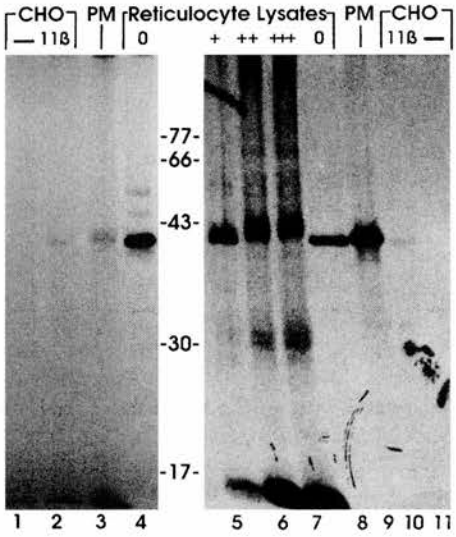


Figure 3 Labelling of native, expressed and *in vitro* translated 11β-HSD2 protein

Lanes 1–3 and 9–11: affinity labelling with [^{3}H]corticosterone of native human placental 11β-HSD2 from placental membrane fractions [PM; lanes 3 and 9; 20 and 80 μg of protein loaded respectively, from 25000 ***g*** pellet (heavy microsomal and mitochondrial fraction)] and from homogenates of CHO cells (160 mg of protein/lane) transfected with 11β-HSD2 cDNA (lanes 2 and 10) or with vector only (lanes 1 and 11). Lanes 4–8, products of coupled *in vitro* transcription/translation of 11β-HSD2 cDNA (subcloned into pGEM11zf) in rabbit reticulocyte lysates labelled with [^{35}S]methionine in the presence of various amounts of microsomes [indicated above lane: 0; + = 1.5 μl; ++ = 3 μl; +++ = 4.5 μl (excess)]. The lysate volume loaded in lanes 4–8 was 0.5, 1, 1, 1 and 0.1 μl respectively. The volume loaded was varied to facilitate size comparison of the translated products and was needed to counter the decrease in translation in the presence of higher microsome concentrations. The positions of protein standards ($10^{-3} \times M_r$) are indicated.

microheterogeneity similar to that observed with other steroid-metabolizing enzymes, notably 3α-HSD (98.8% identity) [40,41] and 3β-HSD (rat 3β-HSD2 being 99.2% and > 93% identical to 3β-HSD2 male liver variant [42] and 3β-HSD1 [43] respectively). The finding of polymorphisms that affect the 11β-HSD2 protein sequence is clearly of considerable interest, especially if they are associated with a difference in steroid metabolism. Placental 11β-HSD2 has a K_m for corticosterone (see below; Table 1) virtually identical to that of the 1000-fold purified native 11β-HSD2 from placenta [9]. The K_m reported for the renal clone is 3-fold lower (~ 4.4 nM), and it could be that amino acid substitutions at residue 148 cause subtle alterations in steroid-metabolizing activity by affecting the predicted α-helical region of which it is part.

Expression studies with human placental 11β-HSD2 cDNA

Expression of 11β-HSD2 protein

Expression of p11β2 in CHO cells produced abundant 11β-HSD2 enzyme activity (see below), accompanied by the expression of an ~ 40000-M_r protein which could be affinity-labelled with corticosterone (Figure 3, lanes 2 cf. 1 and 10 cf. 11), in a similar manner to the affinity labelling of 11β-HSD2 from crude placental subcellular fractions (Figure 3, lanes 3 and 9). Coupled *in vitro* transcription/translation of 11β-HSD2 in rabbit reticulocyte lysates (without microsomal processing) also produced a protein (Figure 3, lanes 4 and 8) of similar size to 11β-HSD2 in placenta or expressed in CHO cells. Thus activity in these tissues appears not to require major cleavages or large covalent attachments. Addition of canine pancreatic microsomes resulted in a small size increase and broadening of the protein band, suggesting that co-translational processing, possibly involving glycosylation, was occurring (Figure 3, lanes 4–8). Addition of increasing amounts of microsomes also resulted in a reduced efficiency of translation (a standard finding with TNT lysates); accordingly more sample was loaded (to facilitate size comparison), and a minor band at ~ 31000-M_r became visible. It is unclear if this is a different, minor, translation product or the result of 11β-HSD2 proteolytic cleavage (processing or degradative) occurring at a low level in the presence of these microsomes. Clearly a range of processed states of 11β-HSD2 is demonstrated and may indicate the possibility of tissue-specific co-translational processing depending on the activities within host tissue microsomes. If cleavage to an ~ 31000-M_r form occurs, this is likely to affect enzyme structure/function and possibly its subcellular localization. Finally, although native placental 11β-HSD2 is most likely to be located in microsomes, this is not confirmed by the presence of a classical C-terminal microsomal retention motif [44,45], in contrast to the closely related human 17β-HSD2 [35].

Characteristics of expressed enzyme activity

Expression of the 11β-HSD2 cDNA in CHO cells produced high-affinity 11β-HSD activity which had the expected characteristics of 11β-HSD2 activity in placenta. This was exclusively NAD^+-dependent in cell homogenates (12 nM corticosterone and 0.15 mg of protein/ml), showing 49% conversion with 400 μM NAD^+, whereas 400 μM $NADP^+$ produced no increase over assay with no added cofactor (1.8%). No 11β-reductase activity was detected in homogenates of 11β-HSD2-transfected CHO cells, or in the medium of the intact cells (which metabolized 99% of 25 nM [^{3}H]corticosterone in 24 h). 11-Dehydro products were the only metabolites detected by HPLC. Thus after a 1 h incubation of [^{3}H]steroid (12 nM) with recombinant 11β-HSD2

Figure 2 cDNA and predicted amino acid sequence of human placental 11β-HSD2

Top panel: the cDNA numbering (−133 to +1764; shown on the left of the DNA sequence) sets +1 at the start of the predicted coding region (note that the 'no base zero' convention is followed). The ATG initiation codon and polyadenylation motif are double-underlined. The broken line encloses the 531 bp CtBb fragment identified by RT-PCR (Figure 1, lane 5). Boxed sections of predicted amino acid sequence are those of the 10 peptides sequenced from tryptic digests of purified human placental 11β-HSD2; peptide nomenclature (small boxes) and numbering of the amino acid sequence are to the side of the sequence (amino acid sequence numbers are underlined). Circled residues are Cys-227 (unidentifiable residue on amino acid sequencing of peptide C) and Asn-394 (the only potential N-glycosylation site in 11β-HSD2, probably unused in placenta). Middle panel: predicted secondary structure of human placental 11β-HSD2 and that derived from X-ray crystallography for 3α,20β-HSD from *Streptomyces hydrogenans* [33]. Segments represented as white (α-helix, above line; β-sheet, below line) and black (loops; close to line) are of the greatest certainty; grey segments are of moderate certainty and segments represented by a flat line cannot be predicted with even moderate certainty. Bottom panel: alignment of 12 enzymes with sequence and functional similarity to human 11β-HSD2. On the left, percentage amino acid identity with regions I–IV of human 11β-HSD2 is indicated by larger-type numbers (small type indicates amino acid residue numbers at the start of regions). On the right, alignment of three motifs highly conserved in SCADs, with $(X)_n$ indicating that *n* amino acids separate the first residues of the adjacent motifs (inclusive). Enzymes are: h11β2 and s11β2, human and sheep 11β-HSD type 2 respectively; h17β1/2/3, human 17β-HSD types 1, 2 and 3; p17β4, pig 17β-HSD type 4; b11retdh, bovine 11-*cis*-retinol dehydrogenase; h3hbdh, human 3-hydroxybutyrate dehydrogenase; h11β1, human 11β-HSD type 1; C.s7αhsd, 7α-HSD from *Clostridium sordellii*; 3α20βhsd, 3α,20β-HSD from *Streptomyces hydrogenans*; pCarbred, pig carbonyl reductase; h3β2, human 3β-HSD type 2 (the adrenal/ovarian isoform).

Table 1 Kinetic parameters of 11β-HSD2 activity in cells transfected with 11β-HSD2 cDNA

All values are based on at least 10 steroid concentrations; $n = 5$ at each concentration for corticosterone and $n = 2$ for cortisol and dexamethasone. Aldosterone was not metabolized.

Glucocorticoid	K_m (nM)	$V_{max.}$ (pmol/h per mg of protein)
Corticosterone	12.4 ± 1.5	8.0 ± 0.7
Cortisol	43.9 ± 8.5	4.8 ± 0.7
Dexamethasone	119 ± 15	3.7 ± 0.5
Aldosterone	not metabolised	

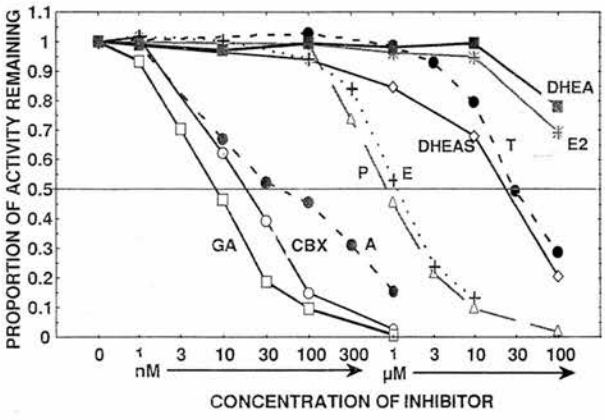


Figure 4 Studies on inhibition of 11β-HSD2

The extent to which a range of compounds inhibit the conversion of 12 nM [^{3}H]corticosterone by recombinant 11β-HSD2 is shown. GA, glycyrrhetinic acid; CBX, carbenoxolone; A, 11-dehydrocorticosterone; P, progesterone; E, cortisone; E2, oestradiol; DHEAS, DHEA sulphate. Reactions were in the presence of 400 μM NAD^+, using homogenates (0.15 mg of protein/ml) of cells transfected with 11β-HSD2 cDNA. Values are means of four determinations, with S.E.M.s < 4%.

(0.5 mg of homogenate protein/ml), > 95% of corticosterone, 63% of cortisol and 17% of dexamethasone were metabolized, whereas aldosterone was unaltered. There was no evidence of 5α- or 5β-reductase activity encoded by p11β2.

Recombinant human placental 11β-HSD2 has a high affinity for glucocorticoid substrates, especially corticosterone (Table 1). At corticosterone concentrations higher than 80 nM there appeared to be a degree of product/substrate inhibition. Examination of 11β-HSD2 inhibition (IC_{50} values) by a range of steroids showed potent inhibition by glycyrrhetinic acid (11 nM) and carbenoxolone (18 nM), with significant inhibition by 11-dehydrocorticosterone (45 nM), progesterone (0.8 μM) and cortisone (1 μM), whereas there was little effect (> 30 μM) with dehydroepiandrosterone (DHEA), DHEA sulphate, testosterone and oestradiol (Figure 4). Affinity labelling studies (results not shown) on transfected CHO cells indicated the same rank order of potency in labelling [corticosterone ≫ cortisol ≫ dexamethasone (aldosterone = 0)] as indicated by their K_m values (Table 1) as 11β-HSD2 substrates. A rank order of affinity as substrate/inhibitor is thus suggested for placental 11β-HSD2 of: glycyrrhetinic acid ≈ carbenoxolone ≈ corticosterone > 11-dehydrocorticosterone ≈ cortisol > dexamethasone > progesterone ≈ cortisone ≫ DHEA sulphate ≈ testosterone ≈ oestradiol ≈ DHEA ≈ aldosterone. This rank order is very similar to that determined for the type III corticosterone receptor sites [46,47] and suggests that these sites are related to 11β-HSD2, but whether they are actually due to the 11β-HSD2 protein remains unclear. In late human gestation progesterone levels often rise to 0.3–0.5 μM in the mother and > 1 μM in the fetus [48]. Thus the IC_{50} of progesterone for 11β-HSD2 (0.8 μM) may signify a potential influence of progesterone on corticosteroid action during late pregnancy.

Distribution of 11β-HSD2 mRNA and protein in tissues

11β-HSD2 mRNA distribution

Northern analysis revealed a hybridizing ~ 1.9 kb transcript in the placenta, aldosterone target tissues (kidney, colon, parotid,

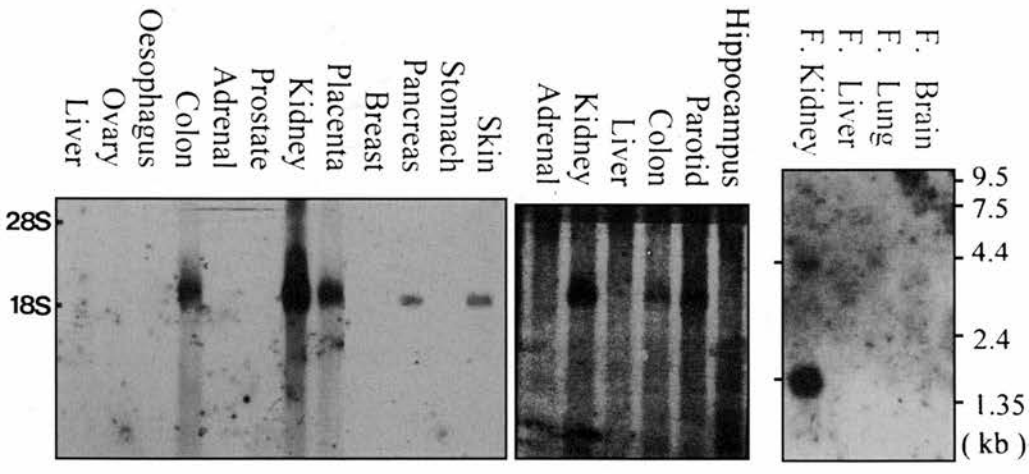


Figure 5 Northern hybridization of 11β-HSD2 cDNA in human tissues

Hybridization of RNA from a range of human adult (total RNA) and fetal [F.; highly purified poly(A) RNA] tissues with a random-primed human placental 11β-HSD2 cDNA probe (bases 217–1737; see Figure 2). Positions of fetal RNA markers and of 18 S and 28 S markers are indicated. Check-marks on left of the fetal-tissue blot correspond to the two mRNA species hybridizing, at ~ 1.9 and ~ 4 kb.

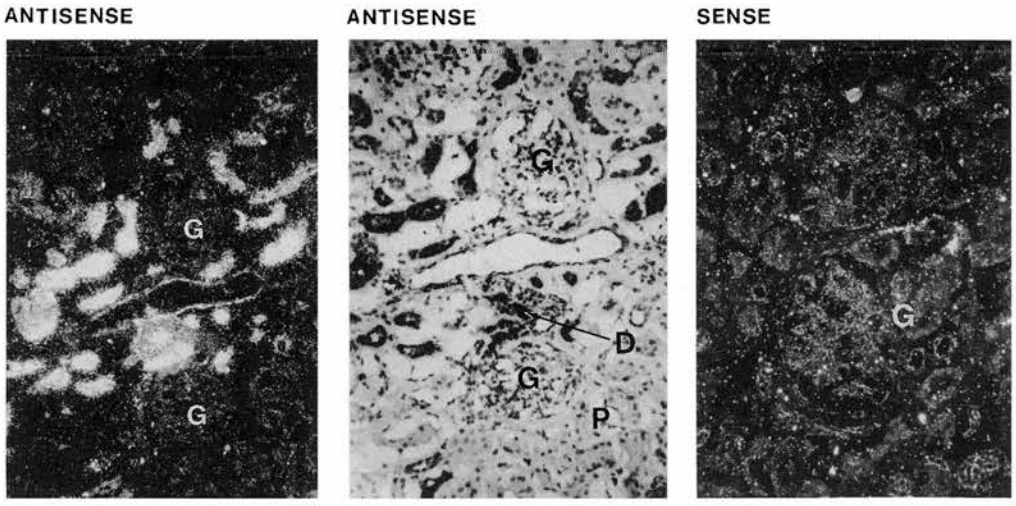


Figure 6 11β-HSD2 *in situ* hybridization in human kidney

In situ hybridization of sections of human kidney to human placental 11β-HSD2 cRNA probes (sense and antisense to CtBb fragment; dashed box in Figure 2). The middle panel shows a light field (silver grains = dark), whereas the other panels are dark field (silver grains = white). The left and middle panels are of the same section, the latter at a slightly higher magnification, whereas the right-hand panel is of a neighbouring section from the same kidney slice (at higher magnification). The following are labelled: G, glomerulus; D, distal convoluted tubule; P, area with many proximal convoluted tubules. Clearly 11β-HSD2 mRNA is abundant in the distal nephron.

skin), pancreas and fetal kidney (Figure 5). There was also hybridization to mRNA of a larger size (~ 4 kb) in fetal kidney. Under these conditions there was no detectable hybridization to RNA from liver, oesophagus, stomach, ovary, prostate, breast, fetal lung, fetal liver or fetal brain, or from adult human brain sub-regions (frontal cortex, cerebellum, hippocampus, hypothalamus, pons and medulla; results not shown). *In situ* hybridization with 11β-HSD2 cDNA on normal human adult kidney (Figure 6) showed abundant 11β-HSD2 mRNA expression restricted to the distal nephron (distal convoluted tubule, cortical collecting duct and medullary collecting ducts), with no specific hybridization in other regions (glomerulus, proximal tubule, etc.). Expression in the distal convoluted tubule extended to loops participating in juxtaglomerular complexes. There was no hybridization in sense controls. There was also abundant expression of 11β-HSD2 mRNA in human placental trophoblast (results not shown).

The nature of the ~ 4 kb 11β-HSD2-hybridizing transcript in fetal kidney is unclear. Interestingly, flanking duplications involving the genes encoding human steroid-metabolizing enzymes are common (e.g. 17β-HSD type 1, the 3β-HSD family, 5α-reductase type 1, 21-hydroxylase and 17β-HSD type 4), and duplicates are often transcribed, either as separate transcripts or, in the case of 17β-HSD4, as a large transcript traversing the apparent gene duplication.

Immunohistochemistry

Antisera raised to a synthetic 11β-HSD2 peptide (residues 370–383) coupled to keyhole limpet haemocyanin identified a single strong protein band at ~ 40000-M_r in Western blots of human placenta and kidney tissue extracts (Figure 7a). This was also detected in CHO cells transfected with 11β-HSD2 cDNA, but not in untransfected controls. This band was not observed when the antisera were preabsorbed with 11β-HSD2-(370–383)-peptide. An additional, much weaker, band was seen at ~ 70000-M_r in some kidney extracts. Immunohistochemistry on human placenta revealed dense immunostaining of the trophoblast (in the syncytiotrophoblast layer), but not the decidua (Figure 7b). Very dense immunostaining in adult human kidney (Figure 7c) was seen localized to the distal nephron (distal convoluted tubule including juxtaglomerular loops, cortical and medullary collecting ducts). In both tissues the immunostaining was abolished by preabsorption with 11β-HSD2-(370–383)-peptide (Figures 7b and 7c). Neither tissue showed any immunostaining using preimmune serum (results not shown).

In situ hybridization and immunohistochemistry demonstrated abundant 11β-HSD2 mRNA and protein in kidney in the location expected (distal nephron) for 11β-HSD2 to confer aldosterone selectivity on mineralocorticoid receptors. 11β-HSD2 expression in the distal convoluted tubule extends into loops participating in juxtaglomerular complexes. In placenta 11β-HSD2 expression was abundant in the syncytiotrophoblast lining the placental villi, thus being at the very interface between the fetal tissue and maternal blood, which flows in the intervillus spaces. This is exactly the location which would allow 11β-HSD2 the greatest influence over the passage of maternal glucocorticoid to the fetus.

Developmental expression

Two studies, using tissue slices from fetuses at 10–20 weeks gestation [49] or adding NAD^+ to tissue homogenates from 16–19-week fetuses [12], have found substantial levels of 11β-dehydrogenase activity in almost all human fetal tissues (including kidney, lung and brain). Fetal liver was found to have net 11β-reductase in the first study and an NAD^+-dependent 11β-dehydrogenase in the second. We found no 11β-HSD2 hybridization in fetal lung, liver or brain. As 11β-HSD1 mRNA is reported absent at least from fetal lung and liver [12], it is possible that a third 11β-HSD isoform is expressed in the fetus. However, as our samples were from fetuses slightly later in gestation it is possible that 11β-HSD2 expression, while present

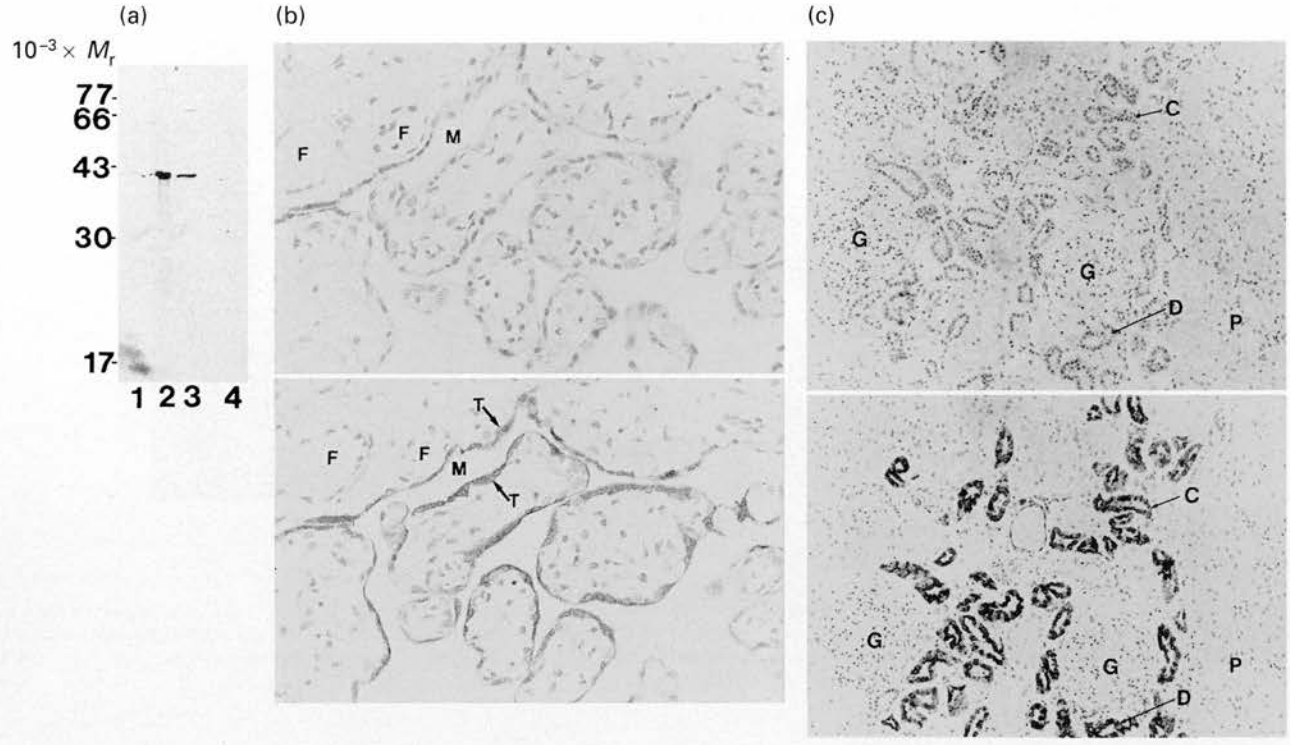


Figure 7 Studies with antisera to human placental 11β-HSD2-(370–383)-peptide

(**a**) Western blot using anti-11β-HSD2 antisera and blotted tissue subfractions (25000 *g* pellet; mainly heavy microsomes and mitochondria) from: CHO cells transfected with (lane 1) and without (lane 4) 11β-HSD2 cDNA, human placenta (lane 2) and human adult kidney (lane 3). Positions of protein standards are indicated. (**b**) and (**c**) Immunohistochemistry on (**b**) human placenta and (**c**) human adult kidney with haematoxylin counterstaining (stains nuclei). Lower panels, antisera to human placental 11β-HSD2-(370–383)-peptide; upper panels, control with antisera preabsorbed with the 11β-HSD2-(370–383)-peptide. Panel (**b**) shows a high-power view of placental villi cut cross-sectionally. Fetal blood circulates in fetal capillaries (two are marked F) within the villi, maternal blood occupies the intervillus space (marked M), and abundant 11β-HSD2 expression is localized in the syncytiotrophoblast layer (T) which intervenes between the fetal and maternal circulations. Panel (**c**) shows a medium-power view of human kidney cortex. The following are labelled: G, glomerulus; D, distal convoluted tubule; P, area with proximal tubules; C, cortical collecting duct. Clearly the 11β-HSD2 protein is abundant in the distal nephron.

during early gestation, is switched off in many fetal tissues (the kidney being a clear exception) at mid-gestation (specifically before 22–23 weeks in lung, 22–26 weeks in liver and 21–26 weeks in brain). Indeed, our preliminary studies on mouse development support this pattern of expression (R. W. Brown, R. Diaz and J. R. Seckl, unpublished work). The mechanism of such developmental control is unknown but, interestingly, it seems that a CpG island may be associated with 5′ regulatory regions of the 11β-HSD2 gene, since: (1) the CpG island extends to the very start of the 11β-HSD2 clone, (2) from the 11β-HSD2 transcript size on Northern blots it appears that the p11β2 clone must be approximately full length (although we have not precisely mapped the start site) and (3) CpG islands are large (typically > 1 kb) and often include the most 5′ exon as well as upstream sequences [30]. This is intriguing because CpG islands are often methylated in tissues where the gene is not expressed. Silencing of the expression of genes with 5′ CpG islands may be associated with methylation of the CpG island. Whether a regulatory influence of this kind occurs in the 11β-HSD2 gene remains to be elucidated. Work on baboon pregnancy suggests that the 11β-HSD barrier to maternal glucocorticoids only becomes firmly established after mid-gestation [48,50]; prior to this fetal tissues may express 11β-HSD2 and 'protect themselves' from high maternal glucocorticoid levels. As the placenta takes over the protective role, fetal tissues adopt a much more 'adult' 11β-HSD2 expression pattern and fetal adrenal activity may begin to dictate their glucocorticoid exposure.

Conclusion

The human placental cDNA that we have isolated encodes an enzyme with the expected characteristics of 11β-HSD2 activity in placenta. The location of 11β-HSD2 mRNA and protein in human tissues supports its proposed key roles as a modulator of glucocorticoid action and mineralocorticoid receptor specificity and as a major determinant of the glucocorticoid exposure of the developing fetus.

It is unlikely that a direct X-ray-crystallography-derived structure will be available in the near future for 11β-HSD2 (which is an intrinsic membrane protein). We have presented its predicted secondary structure in some detail. This will be of particular relevance, as mutations are sought in the protein which may explain: (i) the dramatic loss of 11β-HSD2 enzyme activity that appears to occur in the syndrome of apparent mineralocorticoid excess (SAME), (ii) the more subtle variations in 11β-HSD activity indicated by the altered urinary glucocorticoid metabolites described in subsets of patients with essential hypertension [51] or polycystic ovary disease [52], or (iii) the impaired placental 11β-HSD2 activity which correlates with reduced birth weight in humans [6].

Finally, two further points remain unexplained. First, why does 11β-HSD2, which metabolizes a hydrophobic substrate using a positively charged cofactor (NAD^+), possess such a positively charged structure? Secondly, if mutations in 11β-HSD2 cause SAME, how do the associated alterations in 5α/5β-reductase activity [53,54] come about, as 11β-HSD2 appears to have neither activity? Clearly further studies of this key enzyme, 11β-HSD2, will not only shed light on such matters but are likely to contribute to a better understanding of the role of corticosteroid physiology in development and the aetiology of hypertension in humans.

Note added in proof. (received 13 November 1995)

After completion of this work the first crystal structure for 17β-HSD1 has been reported [55], showing an N-terminal SCAD region followed by α-helices and a flexible C-terminal region. This is intriguingly similar to the structure predicted above for 11β-HSD2.

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